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SYMPATHETIC MECHANISMS OF SALT-SENSITIVE HYPERTENSION

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ANDREW J. KING

has been accepted towards fulfillment
of the requirements for the

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Major Professor's Signature

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SYMPATHETIC MECHANISMS OF SALT-SENSITIVE HYPERTENSION

By

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ABSTRACT

SYMPATHETIC MECHANISMS OF SALT-SENSITIVE HYPERTENSION

By

Andrew J. King

Compelling evidence indicates that sympathetic nervous system activation contributes to the pathogenesis of human essential hypertension. However, the mechanisms by which sympathetic over-activity chronically increases arterial pressure have not yet been established. A neurogenic rat model of salt-sensitive hypertension, driven by chronic infusion of angiotensin II, was utilized for the purpose of understanding these mechanisms. Classically the renal sympathetic efferents are commonly presented as the only ones capable of influencing the long-term level of blood pressure. However, in my studies a novel role of sympathetic innervation to the high capacitance splanchnic organs in chronically increasing arterial pressure was established. In addition, cyclooxygenase derived inflammatory products appear to be critical intermediates in the pathway that mediates sympathetic nervous system activation by angiotensin II in animals fed a high salt diet. The findings of this thesis suggest that the sympathetic nervous system may represent an attractive therapeutic target in the treatment of salt-sensitive hypertension.

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supported me unconditionally allowing me to follow my dreams. For that I am eternally grateful.

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LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
AngII	angiotensin II
APP	arterial plateau pressure
AP	arterial pressure
AT_1	angiotensin II type 1 receptors
AT_2	angiotensin II type 2 receptors
BP	blood pressure
BV	blood volume
CNS	central nervous system
CO	cardiac output
CG	celiac ganglion
CGx	celiac ganglionectomy
CV	cardiovascular
CVP	central venous pressure
CGx	celiac ganglionectomy
DBP	diastolic blood pressure
DOCA	deoxycorticosterone acetate
EPI	epinephrine
HCT	hematocrit
HR	heart rate
HTN	hypertension
im	intramuscular

ip	intraperitoneal
iv	intravenous
JGA	juxtaglomerular apparatus
MAP	mean arterial pressure
MCFP	mean circulatory filling pressure
MSNA	muscle sympathetic nerve activity
NE	norepinephrine
po	orally
PV	plasma volume
RAS	renin angiotensin system
RAAS	renin angiotensin aldosterone system
RDx	renal denervation
SBP	systolic blood pressure
sc	subcutaneous
SHAM	sham operation
SHR	spontaneously hypertensive rat
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNS	sympathetic nervous system
TPR	total peripheral resistance
VPP	venous plateau pressure

INTRODUCTION

1. Human essential hypertension and the long term control of blood pressure

Hypertension (HTN) is defined operationally in the Seventh Report of the Joint National Committee (JNC7) on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (BP) as a persistent elevation in systemic arterial pressure so that systolic BP (SBP) is 140 mmHg or greater and/or diastolic BP (DBP) is 90 mmHg or more (40). The National Health and Nutrition Examination Survey (NHANES) indicates that more than 50 million Americans are hypertensive by these criteria, and the prevalence of HTN is increasing in the United States (111). The World Health Report 2002 estimates that the worldwide prevalence of HTN may be as high as 1 billion people.

1.1. Significance of HTN as a human disease

HTN is a major, independent risk factor for heart attack, heart failure, stroke, chronic kidney disease, peripheral artery disease and retinopathy (40). In fact the relationship between BP and the risk of cardiovascular (CV) events is continuous and even begins within what is considered the normal range. The World Health Organization reports that suboptimal BP (SBP greater than 115mmHg) accounts for 62% of cerebrovascular disease and 49% of ischemic heart disease; and is the number one attributable risk for death in the world (40). Despite widely available and affordable treatments, approximately two thirds of hypertensive patients in the United States have a BP that is not appropriately controlled (111),

clearly indicating the need for a more thorough mechanistic understanding of the pathophysiology of HTN in order to identify new therapeutic strategies.

1.2. Etiology of human HTN

The cause of HTN in human patients is identifiable in only 5-10% of cases (40). Primary or essential HTN refers to the other 90-95% of cases of unknown cause. Although the underlying etiology in essential HTN is unclear, mechanistically it must be related to a defect in the long term regulation of arterial BP. The control of BP involves complex, time-dependent interactions of neural, hormonal and renal regulatory mechanisms (193). Two fundamentally distinct hypotheses have been proposed to explain the long term control of arterial BP (45). One is based on the overriding dominance of the kidneys in the long term control of BP; the other proposes that the sympathetic nervous system (SNS) is ultimately responsible for setting the long term level of BP.

1.3. The Guyton hypothesis of long-term BP regulation

The most well established and widely supported model of long term BP control was developed by Guyton and colleagues. The central tenant of this proposal, illustrated in **figure 1**, is that the chronic renal function curve, which determines the positive relationship between renal perfusion pressure and urinary sodium excretion (the "pressure-natriuresis mechanism"), along with whole body autoregulation ultimately serve to regulate blood volume, cardiac output and the long term level of BP (107). The central hypothesis is that BP is set at a level

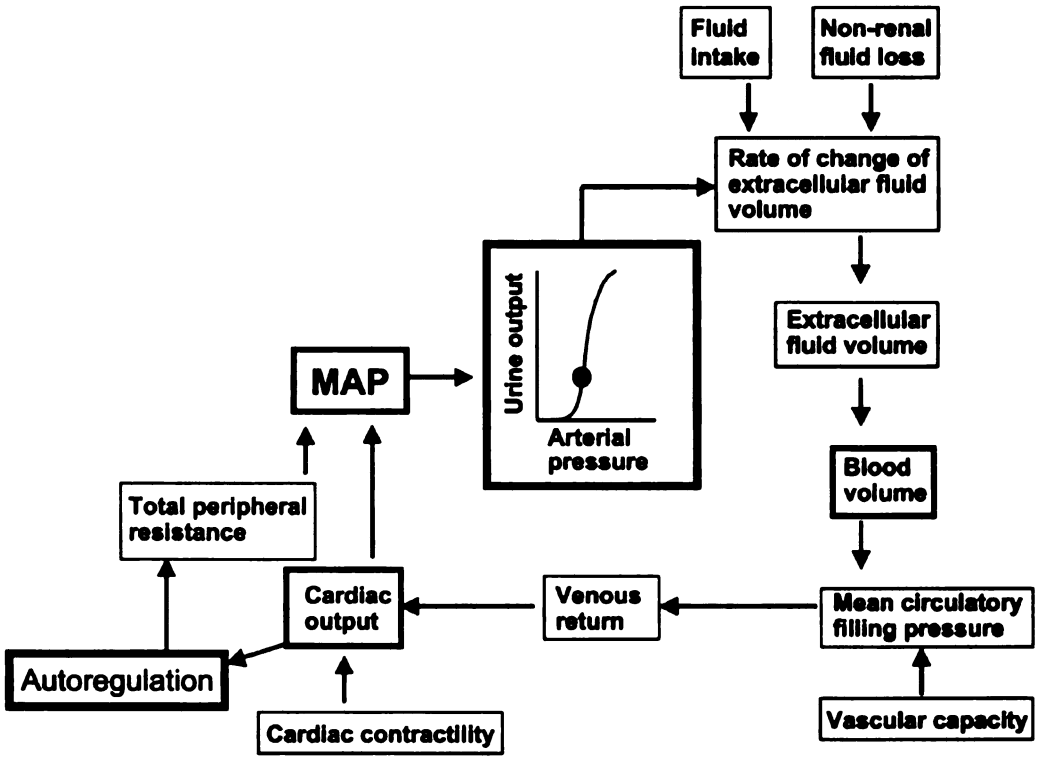


Figure 1: Guyton's model of long-term blood pressure (BP) regulation (107). Central to the model is that BP is ultimately set at a level, determined by the renal function curve, to ensure sodium homeostasis is maintained. Whole body autoregulation is then engaged to protect tissues from overperfusion. Reproduced from figure 1 in Osborn (193). See text for details.

required by the kidneys to excrete a load of sodium (and volume of urine) equivalent to the daily intake (45). HTN is therefore a necessary consequence of a defect in urinary sodium excretion, as a higher level of arterial pressure (AP) is required to restore sodium balance utilizing the pressure natriuresis mechanism. In mathematically modeling the CV system, Guyton assigns the kidneys the characteristic of "infinite gain" (105), meaning pressure-natriuresis is capable of fully correcting any blood pressure disturbance given sufficient time. In fact he indicates that changes in circulatory function cannot cause HTN in the absence of a defect in renal sodium excretory function (105). Based on Guyton's theory a defect in renal sodium excretion results in blood volume expansion, an increase in CO and therefore an increase in AP (45). Whole body autoregulation theory predicts that local controllers within the tissues respond to undefined metabolic signals to set local blood flow to a level to meet metabolic needs and avoid overperfusion, thereby ultimately causing an increase in total peripheral resistance (TPR) (107). An increase in TPR is the typical hemodynamic pattern found in human essential HTN (45).

Despite the compelling argument for the overriding dominance of the pressure-natriuresis mechanism in setting the level of BP in the long term, inconsistencies exist between the Guyton theory and experimental and human HTN. The most notable discrepancy is the fact that blood volume is rarely increased in HTN (225). Elegant studies by two independent groups have also questioned the role of the pressure-natriuresis mechanism under physiological conditions. Bie and

coworkers concluded that "although pressure natriuresis is a powerful mechanism.... it seems possible that it is not operative under normal conditions" (15). Reinhardt and colleagues also indicated that the physiological role of pressure natriuresis is limited and that it is not operational when renal perfusion pressure is changed by -20 to +10% (236). Both groups indicate that neuro-humoral control of sodium excretion predominates and in particular the neural control of renin release is critical (15, 236).

1.4. The neural hypothesis of long-term BP regulation

An alternative to the Guyton hypothesis is that the central nervous system (CNS), by adjusting efferent sympathetic tone provides long term control of BP (104, 193). The mechanism by which the CNS is informed about short-term changes in BP clearly involves mechanical stretch receptors and the baroreflex; however the primary mechanism by which the CNS senses the long-term level of BP is unclear, but appears to predominately involve non-baroreflex pathways (9, 123, 195, 199). A major reason that the importance of the SNS in the long-term regulation of BP is often discounted is the documented adaptation of the SNS to elevations in BP. However, the finding that sympatholytic drugs are among the most effective at chronically lowering BP points to the power of sympathetic control of BP (166). The CNS can theoretically control BP by regulating a number of parallel pathways that ultimately converge to determine the long-term level of AP, as depicted in **figure 2** (193). AP is determined by the product of CO and TPR [MAP = CO X TPR] (45), both of which are strongly influenced by the

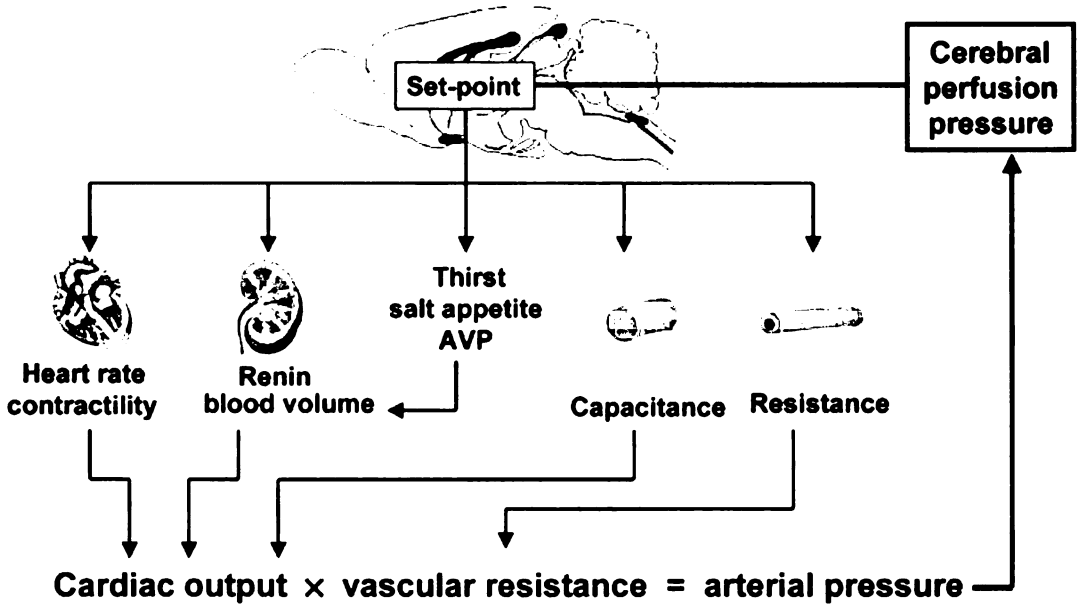


Figure 2: Neural model of long-term BP regulation. Several parallel pathways are engaged to ultimately affect the basic determinants of BP, CO and TPR. Reproduced from figure 2 in Osborn (193). See text for details.

SNS. Cardiac sympathetic efferents activate adrenergic receptors on the heart to accelerate heart rate (HR) and enhance myocardial contractility, both of which contribute to an increase in CO (104). In addition the BP raising actions of SNS activation include a reduction in vascular capacitance, which ultimately determines the distribution of blood volume within the circulation, predominately via an action on the venous system (104). Long-term whole body water balance and subsequently blood volume is also influenced by the SNS, due to an action of renal sympathetic nerves to enhance renal sodium and water retention (104). The SNS also acts directly to increase TPR by causing constriction of the resistance arteries (104). The SNS is also a major regulator of the renin-angiotensin system (RAS) by acting on β_1 receptors in renal juxtaglomerular (JG) cells to increase renin release (45).

Although there are a number of viable candidate mechanisms by which the SNS can chronically regulate AP, **"how an increase in sympathetic activity raises the 24-h mean BP has not been established"** (104). The goal of the work presented in this thesis is to identify mechanisms by which SNS activation can chronically increase AP and cause hypertension.

2. SNS activation in HTN

Considerable evidence suggests that SNS activation may be a common mechanism in both human essential HTN (3, 65, 72, 81, 98, 233) and many

experimental animal models (6, 24, 156, 251) used to study HTN in the laboratory.

2.1. SNS activation in human HTN

The evidence for increased SNS activity in human HTN is compelling. The most convincing evidence comes from direct recordings of single fiber sympathetic nerve activity in human hypertensive patients (103, 176). Multi-unit microneurography techniques and measurements of regional and whole body norepinephrine (NE) spillover also demonstrate increased SNS activity in human HTN (63, 64). Enhanced depressor responses to acute administration of ganglion blocking drugs, and the efficacy of chronic treatment with centrally and peripherally acting sympatholytics in people with HTN also supports increased SNS activity in this population (85, 177). Some studies show hypertensive patients have increased plasma and urinary catecholamines compared to normotensive controls, although this has not been a consistent finding (85). SNS activation is most consistently evident in younger patients during the early stages of HTN (64).

2.2. SNS activation in experimental HTN

Using many complementary techniques including measurements of plasma and urinary catecholamines, microneurography, NE spillover and sympatholytic responses, it appears that SNS activation is also a feature of most experimental animal models of HTN (6, 24, 156, 167, 251). The findings that chemical sympathectomy, CNS lesions and regional sympathetic denervation can

attenuate or abolish many forms of experimental HTN also indicates that SNS activation is critical in the pathogenesis of these models (20, 43, 115, 127, 210). Therefore SNS activation may be a common mechanism of HTN in both human essential HTN and many experimental animal models. It is important to mention that not all hypertensive patients or models exhibit increased SNS activity.

2.3. SNS activators in human HTN

In selecting an experimental animal model to study neurogenic mechanisms of HTN, it is critical to select a factor to drive the model that has been implicated in activating the SNS in human HTN. This will provide the best opportunity for the findings in the experimental model to be reflective of the condition that ultimately needs to be understood; that is human essential HTN. The specific activators of the SNS in human HTN are largely unknown. There is evidence that SNS firing patterns are at least to some extent genetically determined (80, 268, 284). It has also been hypothesized that behavioral and psychosomatic factors, in particular stress, contribute to SNS activation in human HTN (69, 207, 237). Subsequently the "stress hypothesis" of human HTN has been proposed. Obesity and a positive energy balance have been conclusively shown to increase SNS activity (131, 154). The prevalence of obese hypertensive patients is increasing. The underlying CNS mechanism of sympathetic activation in obese hypertensives may differ from that in the lean hypertensive population (153). The pattern of SNS activation in obese hypertensive patients is characterized by recruitment of previously silent fibers, whereas the hallmark of SNS activation in lean

hypertensives is an increased firing rate of single vasoconstrictor fibers (153). The former is thought to reflect baroreflex function, the latter increased CNS drive. Physical inactivity is also thought to contribute to SNS activation in HTN (130). Angiotensin II (AngII) is a humoral factor that has been demonstrated to activate the SNS in human HTN (12, 99).

2.4. AngII as an activator of the SNS in HTN

Reports on the ability of AngII to activate the SNS in humans are somewhat conflicting. Studies show that AngII type 1 (AT1) receptor antagonists significantly reduce muscle sympathetic nerve activity (MSNA) in lean (12) and obese (99) hypertensive humans and that AT1 receptor blockade or angiotensin converting enzyme (ACE) inhibition reduces MSNA in hypertensive patients with chronic kidney disease (185). However Esler's group combined microneurography and radioisotope dilution methodology in a randomized, placebo-controlled crossover study to demonstrate that MSNA and whole body NE spillover were unchanged by AT1 receptor antagonism in human essential HTN, and concluded that the blood pressure lowering actions of AT1 blockade are not related to sympathoinhibition (150). The evidence for AngII mediated sympathoactivation in experimental animals is also controversial. For example Luft and colleagues attenuated chronic AngII HTN in the rat by adrenergic blockade and showed significant increases in splanchnic nerve activity in conscious AngII infused rats instrumented with splanchnic nerve electrodes (167). However Kline showed no significant differences in NE turnover in the

heart, kidney, skeletal muscle or intestine in AngII hypertensive rats (143) indicating that SNS activity was not increased, although depressor responses to ganglion blockade were significantly larger in the AngII infused rats (143). Therefore it appears as though the effect of AngII on SNS activity depends on the specific human population or the experimental conditions under which it is studied. Central to the hypothesis of this thesis is that one of the conditions promoting AngII mediated sympathoactivation is a high salt diet, and potential mechanisms mediating this interaction have been reviewed recently (196).

2.5. Chronic AngII-salt HTN: A model of neurogenic HTN

"Although evidence that the brain regulates the 24-h average BP and contributes to the hypertensive process is very persuasive, the mechanisms are not well understood." (104). The purpose of the work presented in this thesis is to explore the mechanisms by which the SNS can regulate the 24-hour average BP and contribute to the hypertensive process. Sympatholytic drugs and agents that inhibit the formation or action of AngII are effective in treating many patients with essential HTN (40). This implicates both the SNS and AngII in the pathogenesis of human HTN. Therefore an experimental model of neurogenic HTN, driven by AngII and salt, may provide insight into possible mechanisms of human HTN.

3. The renin-angiotensin system

The renin-angiotensin system (RAS) is an endocrine system important in regulating AP and blood volume. The classical RAS is depicted in **figure 3**.

3.1. RAS activation

RAS activation occurs as a result of a fall in AP or reductions in renal perfusion pressure and is initiated by renin release from the juxtaglomerular (JG) cells in the renal afferent arterioles. Decreased systemic BP is sensed by arterial baroreceptors which signal medullary control centers to increase sympathetic outflow to the JG apparatus (JGA) to increase renin release by activation of β_1 adrenoreceptors. Stretch receptors within the JGA also sense decreased distension associated with reduced renal perfusion pressures and increase renin release by the unusual mechanism of lowering intracellular Ca^{2+} concentrations. Finally decreased distal tubular salt delivery is detected by the macula densa, a modified plaque of sensory cells, and a signal (thought to be adenosine) is sent to the JG cells to increase renin release into the systemic circulation (95)

Renin is an enzyme that proteolytic cleaves circulating, angiotensinogen, which is synthesized in the liver, to form the decapeptide angiotensin I (AngI). Angiotensin converting enzyme (ACE) is a vascular endothelium ecto-enzyme, principally located in the pulmonary circulation, which cleaves AngI to form the octapeptide AngII. AngI has little biologic activity, whereas AngII is a potent physiologically active hormone.

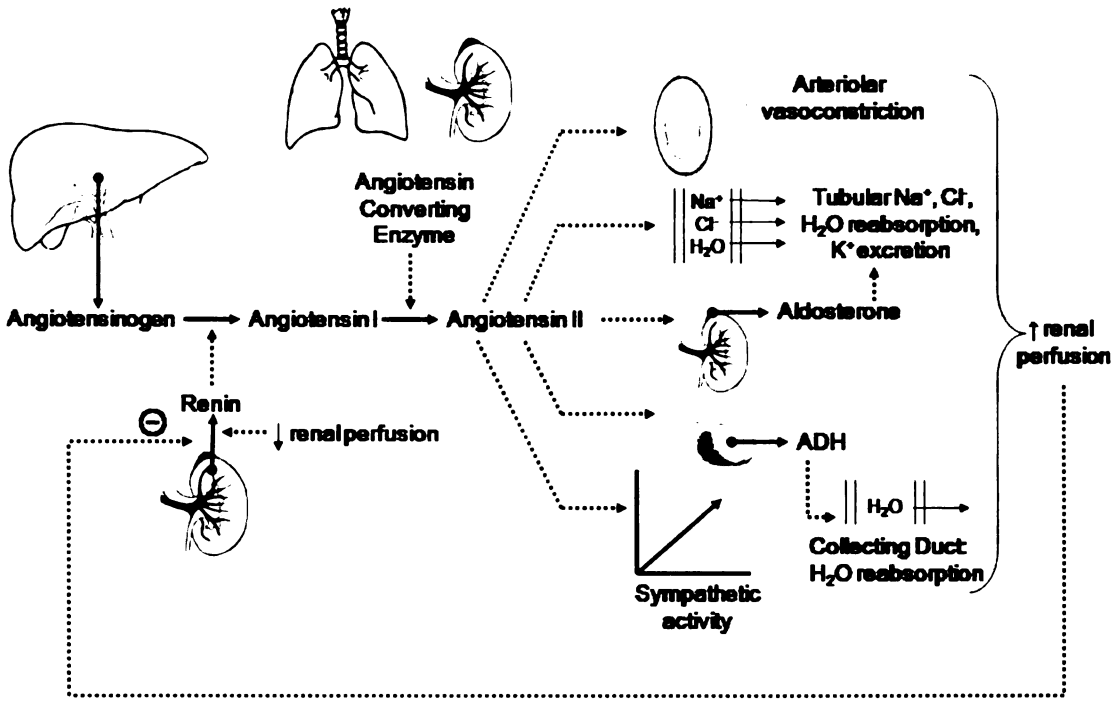


Figure 3: The renin-angiotensin system (RAS). The classic endocrine RAS regulates BP and blood volume by multiple mechanisms including direct arteriolar vasoconstriction, enhanced renal Na^+ and H_2O reabsorption, both directly and via stimulating aldosterone and ADH release, and by activating the SNS. See text for details.

3.2. The actions of the RAS to increase BP

There are two well characterized receptors for AngII, AT_1 and AT_2. The classic actions of AngII are mediated by activation of AT_1 receptors and include systemic vasoconstriction, enhanced renal sodium retention and increased aldosterone and arginine vasopressin (AVP) release. AT_2 receptors seem to be important in development during fetal life and tend to oppose the actions of AngII on AT_1 receptors in adult life (95). ACE inhibitors and angiotensin receptor blockers (ARBs) are both effective in the treatment of human HTN indicating that overactivity of the RAS is likely involved in the mechanism of increased AP (40).

In addition to the well characterized classic actions of AngII, the evidence for AngII activating the SNS is now extremely compelling. Experimental studies indicate that AngII acts on the brain to increase efferent SNS activity (21, 43, 82, 144), acts on sympathetic ganglia directly to increase postganglionic SNS traffic (168) and acts to facilitate noradrenergic transmission and responsiveness at the neuro-effector junction (42, 208, 226). In addition AngII can increase catecholamine release from the adrenal medulla (56). The adrenal medulla releases both norepinephrine and epinephrine, both of which can activate adrenergic receptors on blood vessels and the heart to increase BP.

Advances have been made in the elucidation of the central pathways involved in mediating the sympathoexcitatory effect of AngII, suggesting that systemically delivered AngII likely activates critical circumventricular organs (21, 43, 82, 144)

with efferent projections to brain centers known to influence SNS activity (144). Oxidative stress may mediate this central sympathoexcitatory effect (30). However, there is still substantial uncertainty as to the critical peripheral target and the hemodynamic response to this increased SNS activity. A goal of this thesis was to identify the important peripheral mechanisms by which SNS activation can contribute to the pathogenesis of chronic AngII-salt HTN.

4. Salt-sensitive HTN

The concept of salt-sensitivity of BP refers to the phenomenon that a high dietary salt intake can significantly increase BP in some individuals, but have little to no effect on BP in others (26). Salt-sensitivity is more common in hypertensive individuals and may play a role in the pathogenesis of HTN (270). In fact the risk of adverse CV events is more than three times higher in salt-sensitive patients (183). Dietary sodium restriction, not to exceed 2.4 grams per day, is among the recommendations of the JNC7 report on the prevention, detection, evaluation, and treatment of high BP (40). The mechanisms involved in salt-sensitivity have not been determined.

4.1. Renal mechanisms of salt-sensitivity

Most research efforts to identify the mechanism of salt-sensitivity have focused on the kidney because of its central role in regulating salt and water homeostasis. Guyton's model of the circulation suggests that reductions in the steepness of the pressure-natriuresis relationship due to a defect in renal sodium

excretion will impart salt sensitivity on AP (109). In fact Guyton suggests that HTN is a necessary consequence of the reduced ability of the kidney to excrete a salt load (109). The higher AP in HTN is therefore necessary to promote natriuresis in order to maintain sodium balance (109). In support of this concept, the pressure natriuresis curve is significantly less steep in salt sensitive patients, suggesting a defect in sodium excretion (25, 138). Guyton hypothesizes that sodium retention, which is accompanied by volume expansion, increases CO and AP. The most compelling evidence for a renal basis of salt-sensitivity is from renal cross-transplantation studies in genetically hypertensive rats (27). HTN and salt-sensitivity of BP has been shown to be transferable with the hypertensive kidney in Dahl salt-sensitive rats and the spontaneously hypertensive rat (SHR) (14, 49-51). This suggests that the genetic basis of salt-sensitivity resides in the kidney.

Blood volume however, is typically unchanged or decreased in established HTN, and the hemodynamic profile is usually an increase in TPR not CO (225). This appears inconsistent with Guyton's theory. As described earlier, the proposed mechanism of whole body autoregulation (105), whereby increased CO results in tissue hyperperfusion and an autoregulatory vasoconstriction leading to a sustained increase in TPR with CO returning to normal, was historically used to explain the discrepancy. However, a recent examination of the effect of salt loading on BP, sodium balance, plasma volume, CO and TPR over a closely observed (every 4 hours) and extended time course (10 days) in salt-sensitive

and salt-resistant black hypertensives, challenges Guyton's mechanistic concept of salt-sensitivity (234). Sodium loading caused no greater increases in sodium balance, body weight, plasma volume and CO in salt-sensitive patients, compared to salt resistant hypertensives. Instead, the progressively increasing BP in response to salt loading in salt-sensitive individuals was the result of differential responses in TPR, compared to salt-resistant patients (234). This conclusively demonstrates that in this population salt-sensitivity of BP is not due to a defect in sodium excretion and volume related mechanisms, but to alterations in systemic vasoconstrictor tone.

4.2. Neural mechanisms of salt-sensitivity

A number of studies have also implicated SNS overactivity in the pathogenesis of salt-sensitive HTN. It is well established that chronic low-dose AngII infusion is a salt sensitive model of HTN, and there is evidence to suggest that the additional hypertensive effect of salt is mediated by SNS activation (18, 19, 194, 231, 259). Also it has recently been shown that the salt-sensitivity of DOCA-salt hypertension is not mediated primarily by volume related mechanisms, but rather is due to an increased sensitivity of BP to increments in total body water content (258). Other studies have demonstrated that the effect of salt in the DOCA model is in the brain to stimulate vasopressin secretion and likely activate the SNS (17, 190). While the most compelling evidence for SNS activation in response to salt is described in rats (18, 19, 194, 231, 259), it has also recently been

demonstrated in rabbits (180, 181) using repeated assessment of BP responses to ganglion blockade as an assay for sympathetic pressor activity.

More importantly there is considerable evidence that neurogenic mechanisms play a role in the pathophysiology of salt-sensitivity in human essential HTN (26, 28, 29, 91, 146, 186, 240). Salt-sensitive hypertensive subjects show an abnormal relationship between plasma catecholamines and urinary sodium excretion and these patients also exhibit exaggerated pressor responses to exogenous NE. A recent study measuring spontaneous arterial baroreflex sensitivity demonstrated abnormalities in autonomic control of the cardiovascular system in association with salt-sensitivity, supporting the hypothesis that salt-sensitivity in human HTN is at least in part neurogenically driven (44).

Central to the hypothesis tested in this thesis is that the salt-sensitivity of chronic AngII HTN in the rat is also at least in part neurogenic in origin and mediated by SNS activation.

5. The role of the venous system and splanchnic circulation in HTN

Despite changes in venous function being consistently demonstrated in both human and experimental HTN, the venous system has largely been ignored in the investigation of the pathogenesis of HTN. Traditionally the veins have been viewed as passive conduits for return of blood to the heart. Veins contain approximately 70% of the blood volume and provide the most important reservoir

of blood in the circulation (201). More importantly to BP regulation, the vascular smooth muscle tone of the venous system determines the distribution of this blood volume throughout the circulation.

5.1. The venous system and vascular capacitance in HTN

Vascular capacitance is the volume of blood contained in a vascular segment at a given distending pressure and is largely determined by venous capacitance, because the compliance of veins is many times larger than arteries (201). Reduced venous capacitance is a consistent feature in humans with essential HTN (235) and in many experimental animal models including DOCA-salt hypertension (83), SHR (175, 261), the angiotensin-dependent two-kidney, one clip hypertensive rat (287) and one-kidney, one-clip Goldblatt hypertension (289).

Venous capacitance is most importantly influenced by blood volume, venous smooth muscle tone and venous structural properties (83). Venous structural changes probably make only a minor contribution to changes in venous compliance in essential HTN (174, 288) and blood volume is generally not increased (225). Therefore increases in venous tone account for the reduced venous capacitance and increased "effective blood volume" characteristics of established HTN. That is, hypertensive individuals behave as if they are volume expanded: for example, they exhibit greater increments in CO and natriuresis to acute volume loads, and larger hypotensive responses to diuretic drug treatment.

Reduced vascular capacitance therefore makes a significant contribution to the circulatory physiology of HTN.

The mechanisms responsible for the changes of venous capacitance function in HTN have not been elucidated (83). However sympathetically mediated venoconstriction appears to play an important role in both human HTN (174) and experimental models (83, 175, 283).

5.2. The control of venous capacitance by the splanchnic SNS

Splanchnic veins and venules account for most of the active capacitance responses in the circulation, and are densely innervated by the SNS (100, 102, 222). It has been estimated that sympathetic innervation to the non-hepatic splanchnic organs accounts for approximately half of the total NE released in the entire body (4). Therefore, the SNS, by virtue of its influence on splanchnic venous smooth muscle tone, is the principal factor regulating vascular capacitance (201). Veins have also been shown to be more sensitive to sympathetic activation than arterioles (119). Therefore the venous system represents a plausible target for increased SNS activity in human and experimental HTN, where SNS activation tends to be relatively modest in magnitude (104).

Increased SNS activity to the splanchnic venous system is expected to decrease venous capacitance and cause a venous to arterial translocation of blood

volume. The arterial circulation in the rat is approximately 60 times less compliant than the venous system (288). A venous to arterial translocation of only a small volume of blood, in the presence of an impairment in renal excretory function, will significantly increase AP (106, 108, 172). This increase in AP can initiate a series of events, including increased myogenic arteriolar constriction and arterial expression of L-type calcium channels (209, 243), that facilitate the maintenance and progression of elevated AP by increasing TPR.

5.3. Targeting the splanchnic SNS as a therapeutic strategy in HTN

A recent study demonstrated that vascular resistance increases in the hepatosplanchnic circulation before any other bed in humans with borderline HTN (248) indicating the splanchnic SNS may be important in the pathogenesis of human HTN. Historically, surgical thoracolumbar splanchnicectomy, which destroys sympathetic innervation to the splanchnic bed, was extremely effective in lowering AP and prolonging survival times in patients with essential HTN (242), in particular those refractory to medical management (275). Consistent with this, a recent study in humans showed that poorly controlled HTN was markedly improved in patients after bilateral T_3 endoscopic sympathetic block (41). Although the mechanism of the effect is unclear, it is possible that the BP lowering effects of this procedure were mediated through inhibition of splanchnic sympathetic nerve activity.

5.4. Mean circulatory filling pressure: an index of venous tone in vivo

Mean circulatory filling pressure (MCFP) is the pressure measured in the vasculature immediately following cardiac arrest, after pressures in all parts of the circulation are made to equilibrate (110). MCFP represents the effective driving force for venous return to the heart (110). The major determinants of MCFP are compliance of the venous system and blood volume (288). MCFP is therefore considered the best methodology for the determination of whole body venous tone *in vivo* (201).

In this thesis repeated measures of MCFP in conscious, undisturbed rats were used to investigate sympathetically mediated changes in venomotor tone during the establishment and maintenance of chronic low-dose AngII-salt HTN. In particular the role of the splanchnic SNS in mediating venous responses to AngII was assessed.

6. Central hypothesis

The work presented in this thesis is based on the central hypothesis that systemically delivered AngII acts on osmotically sensitized brain centers to activate the SNS in a regionally and temporally differentiated manner. Specifically, chronic infusion of AngII causes HTN in rats, in part, by increasing SNS activity to the high-capacitance splanchnic organs, and this effect is dependent on dietary salt intake. The central hypothesis is depicted in **figure 4**.

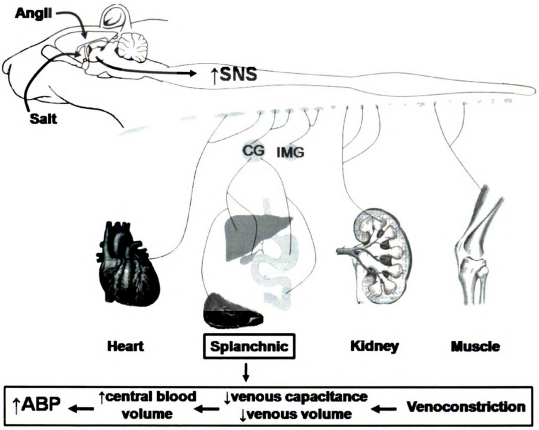


Figure 4: Central hypothesis - AngII acts centrally, in a manner potentiated by a high salt diet, to activate the SNS in a regionally heterogeneous fashion. It is hypothesized that chronic infusion of AngII causes HTN, at least in part, by increasing SNS activity to the high-capacitance splanchnic organs. Sympathetically mediated venoconstriction will likely contribute to a sustained increase in BP by causing a shift in the distribution of blood volume to the less compliant arterial circulation.

This central hypothesis was examined by testing a number of specific sub-hypotheses that are presented in chapters of the thesis.

6.1. Specific hypothesis 1

Chronic infusion of AngII causes a sustained increase in BP. The magnitude of this increase is affected by both salt intake and BP measurement method. (CHAPTER 2)

6.2. Specific hypothesis 2

Chronic infusion of AngII increases global SNS activity in a salt-sensitive manner. (CHAPTER 6)

6.3. Specific hypothesis 3

Chronic infusion of AngII increases venous smooth muscle tone by activation of the SNS, in a salt sensitive manner.
(CHAPTER 3)

6.4. Specific hypothesis 4 Selective removal of the splanchnic SNS will attenuate the venous tone changes and increase in BP in chronic AngII-salt HTN. (CHAPTER 4)

6.5. Specific hypothesis 5

Selective removal of the renal SNS will attenuate chronic AngII-salt HTN.

(CHAPTER 4)

6.6. Specific hypothesis 6

Chronic infusion of AngII in rats fed a high salt diet increases splanchnic resistance.

(CHAPTER 5)

6.7. Specific hypothesis 7

Chronic infusion of AngII increases SNS activity to skeletal muscle in a salt-sensitive manner.

(CHAPTER 7)

6.8. Specific hypothesis 8

Selective downregulation of AT1 receptors in the PVN will attenuate chronic AngII-salt hypertension.

(CHAPTER 8)

6.9. Specific hypothesis 9

SNS activation by chronic infusion of AngII is mediated, in part, by inflammatory factors.

(CHAPTER 9)

7. Overall significance of thesis research

Although progress in advancing the concept that SNS overactivity may be involved in the pathogenesis of HTN has been made, the key to neurogenic HTN requires further understanding. Both the critical CNS networks and peripheral SNS efferents have not been defined. This is best put in context by Guyenet in a recent review published in *Nature Reviews Neuroscience* on the sympathetic control of BP (104), where he states "although evidence that the brain regulates the 24-h average BP and contributes to the hypertensive process is very persuasive, the mechanisms are not well understood". Further he indicates that "the sympathetic efferents that innervate the kidneys are commonly presented as the only ones that are capable of influencing the 24-h average BP" (104). The work in this thesis challenges that notion and documents a novel role of the splanchnic SNS in mediating long-term changes in BP. This is particularly timely given that Eisenhofer noted in his recent review on sympathetic nerve function; "due to difficulties of accessibility, the function of the sympathetic nerves innervating the splanchnic organs remains largely hidden from view" (59).

Characterizing the temporal and regional profile of SNS activation in a rat model of chronic AngII-salt HTN will therefore significantly advance the current understanding of the long term control of AP, and identify specific sympathetic mechanisms of HTN.

CHAPTER ONE: METHODS

1. Animals

All protocols were approved by the Michigan State University All University Committee on Animal Use and Care. Normotensive male Sprague-Dawley rats (Charles River Laboratories, Portage, MI) weighing 225-275g at the beginning of the study were used in all experiments. On arrival rats were housed 3 per cage in a temperature- and humidity-controlled room with a 12-hour light/dark cycle and had free access to food and distilled water. Prior to all experiments rats were acclimatized to a 0.4% or 2% NaCl diet (Research Diets, New Brunswick, NJ) for 7 days.

2. General anesthesia

Injectable or inhalational anesthetic agents were used for the induction and maintenance of general anesthesia for all surgical procedures. All rats recovered from anesthesia on a heating pad under close observation.

Injectable anesthesia: The rats were pre-medicated with atropine (0.04 mg/kg IP) and anesthesia was produced with sodium pentobarbital (40 to 50 mg/kg IP).

Inhalational anesthesia: General anesthesia was induced in an induction chamber using 4% isoflurane in oxygen, and maintained by 2% isoflurane in oxygen delivered by nose cone.

3. Analgesia and antimicrobial prophylaxis

Antimicrobial prophylaxis and post-operative analgesia was achieved by administration of ticarcillin-clavulanate (200 mg/kg IP) and enrofloxacin (5 mg/kg IP), and buprenorphine (0.05 mg/kg SC) respectively. Meloxicam (1 mg/kg PO) was administered daily for 3 days for additional analgesia.

4. Standard AngII HTN protocol

After 7 days of dietary acclimatization to either 0.4% or 2% NaCl diet, rats were chronically instrumented with a radiotelemetry transmitter or exteriorized arterial catheter to allow measurement of AP. Following 7 days of recovery and a 7 day control period, an AngII or physiological saline filled osmotic minipump (2ML2, Alzet, Cupertino, CA) was implanted subcutaneously, to deliver AngII (150ng/kg/min) or vehicle for 14 days. During the entire experimental protocol rats were allowed free access to either 0.4% NaCl or 2% NaCl diet and distilled water. The standard protocol is depicted in **figure 5**. Depending on the particular intervention studied, the exact experimental protocol employed varied slightly in terms of the length of the recovery and control periods.

5. Arterial catheterization

Under general anesthesia, either a silicone-tipped catheter, fabricated in our laboratory, or a commercially available tapered polyurethane catheter (RFA-01, Strategic Applications Inc., Chicago, IL) was inserted into the abdominal aorta, just cranial to the aortic bifurcation, via the left femoral artery. The free end of the catheter was tunneled subcutaneously to exit the rat between the scapulae into a stainless steel spring attached to the rat by a loosely fitted rubber jacket (Instech

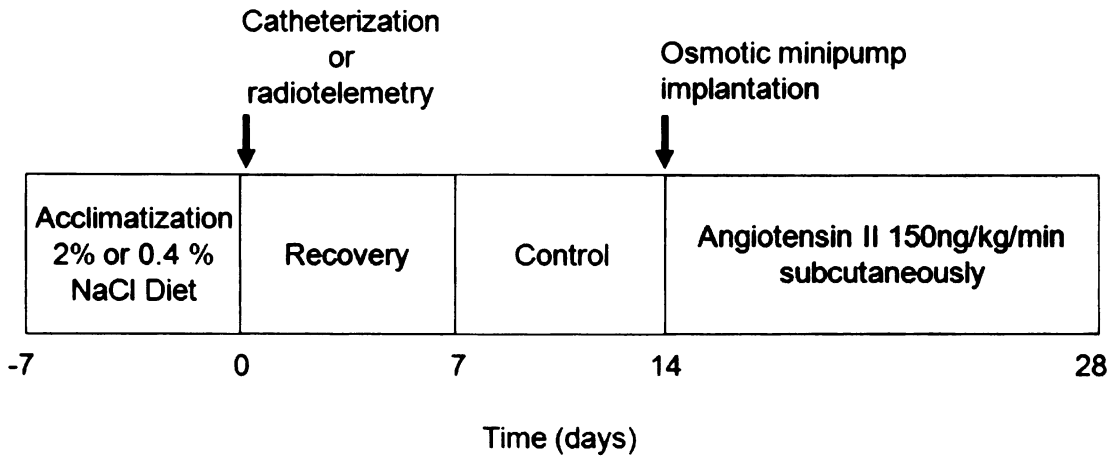


Figure 5: Standard angiotensin II (AngII) infusion protocol. See text for details.

Laboratories, Inc, Plymouth Meeting, PA). The rats were then loosely tethered, using a hydraulic swivel, in individual plastic cages to allow continuous access to all catheters without handling or disturbing. Ticarcillin-clavulanate (200 mg/kg IV) and enrofloxacin (5 mg/kg IV) were administered daily for the duration of the experiment. Vascular catheters were flushed with heparin saline each day.

6. Venous catheterization: femoral

Under general anesthesia, a silicone catheter, fabricated in our laboratory, was inserted into the abdominal vena cava via the left femoral vein. The free end of the catheter was tunneled subcutaneously along with the arterial catheter to exit the rat between the scapulae into a stainless steel spring attached to the rat by a loosely fitted rubber jacket (Instech Laboratories). The rats were then loosely tethered, using a hydraulic swivel, in individual plastic cages to allow continuous access to all catheters without handling or disturbing. Ticarcillin-clavulanate (200 mg/kg IV) and enrofloxacin (5 mg/kg IV) were administered daily for the duration of the experiment. Vascular catheters were flushed with heparin saline each day.

7. Venous catheterization: jugular

Under general anesthesia, a silicone catheter, fabricated in our laboratory, was inserted into the right jugular vein and advanced 3 cm's to the level of the right atrium where it was secured in place. The free end of the catheter was tunneled subcutaneously to exit the rat between the scapulae into a stainless steel spring attached to the rat by a loosely fitted rubber jacket (Instech Laboratories). The

rats were then loosely tethered, using a hydraulic swivel, in individual plastic cages to allow continuous access to all catheters without handling or disturbing. Ticarcillin-clavulanate (200 mg/kg IV) and enrofloxacin (5 mg/kg IV) were administered daily for the duration of the experiment. Vascular catheters were flushed with heparin saline each day.

8. Radiotelemetry transmitter implantation

The TA11-PA-C40 implantable device (Data Sciences International (DSI), Minneapolis, MN) is a small (weight of 9g), cylindrical transmitting device that is implanted subcutaneously into research animals to measure and acquire cardiovascular parameters. This device can sense BP, HR, and physical activity, process this information, and transmit this data from within the animals via radio-frequency signals to a computer for analysis. Under general anesthesia the tip of the transmitter catheter was introduced into the abdominal aorta, just cranial to the aortic bifurcation, through the left femoral artery. The body of the transmitter was placed in a subcutaneous pocket along the caudal-ventral abdomen. Enrofloxacin (5mg/kg IM) was administered once for antimicrobial prophylaxis.

9. Arterial pressure measurement

Exteriorized arterial catheters: AP was determined by connecting the arterial catheter to a pressure transducer (TXD-300; Micro-Med, Louisville, KY) linked to a digital pressure monitor (BPA-400, Micro-Med) that provides measurements of systolic, diastolic and mean pressures and HR at a sampling rate of 1000 Hz. The pressure monitor is connected to a computerized data acquisition program

(DMSI-400, Micro-Med). The pressure transducers were calibrated using a sphygmomanometer and zeroed daily using a column of water placed at the level of the rat's heart. AP and HR were measured at the same time daily for the duration of the experiments and recorded as a 10-30 minute average.

Radiotelemetry: Rats, housed in individual plastic cages, were placed on top a radiotelemetry receiver (RPC-1, DSI). AP and HR were monitored remotely using a commercially available radiotelemetry data acquisition program (Dataquest ART 4.1, DSI). Hemodynamic measurements were sampled for at least 10 seconds every 10 minutes for the duration of the experiment. Data are reported as 24 hour averages.

10. Mean circulatory filling pressure

Catheterization: Under injectable general anesthesia, rats were chronically instrumented with catheters for the measurement of AP and central venous pressure (CVP) and to produce brief circulatory arrest and allow drug administration and blood sampling. Silicone-tipped catheters were inserted into the abdominal aorta, thoracic vena cava and abdominal vena cava through the femoral artery or vein (see **5. arterial catheterization** and **6. venous catheterization: femoral**). A silicone right atrial balloon catheter (Vesta Inc., Franklin, WI) was inserted through the right jugular vein. Optimal balloon catheter positioning was confirmed by a rapid decline in AP (to <30 mmHg within 2 to 3 seconds) and simultaneous rise in CVP (to 6 to 8 mmHg) in response to balloon inflation with 0.25 ml of saline. The catheter was then secured in this location. The ends of all

4 catheters were tunneled subcutaneously and exited the rat between the scapulae into a stainless steel spring attached to the rat by a loosely fitted rubber jacket (Instech Laboratories). The rats were then loosely tethered in individual plastic cages to allow continuous access to all catheters without handling or disturbing the animal. Ticarcillin-clavulanate (200 mg/kg IV) and enrofloxacin (5 mg/kg IV) were administered daily for the duration of the experiment. Vascular catheters were flushed with heparin saline each day and the balloon catheter was briefly inflated daily to prevent adhesions to the atrial wall.

MCFP measurements: MCFP measurements were made according to established methods for the rat (250, 288). Briefly, the right atrial balloon catheter was inflated with 0.25 ml of saline for 5 seconds, resulting in a rapid fall in AP and a simultaneous rise in CVP, both of which quickly plateau. This method results in "trapping" of blood on the low compliance arterial side, preventing full equalization of pressure throughout the circulation. To correct for this, MCFP was computed from arterial plateau pressure (APP) and venous plateau pressure (VPP) using the following formula (288):

$$MCFP = VPP+(APP-VPP)/60$$

11. Blood volume measurements

Plasma volume (PV) was estimated by applying the 10 minute distribution volume of Evans Blue dye method. Hematocrit (Hct) was measured in duplicate

from an arterial blood sample, and blood volume (BV) was computed with the following formula (288):

$$BV=PV/[1-Hct(0.8)/100]$$

12. Celiac ganglionectomy (CGx)

Under inhalational general anesthesia, surgery was performed by a ventral midline laparotomy using aseptic technique. The small intestine was gently retracted to the right side of midline and packed with saline soaked gauze. CGx was performed by locating the celiac plexus in between the aorta, celiac artery and cranial mesenteric artery, dissecting it free from surrounding tissue and removing it. Any additional nerves along these vessels in the area of the celiac ganglion were also dissected free and transected. The small intestine was then returned to the abdominal cavity and the abdominal cavity lavaged with warm saline. SHAM control operation was performed by exposing and visualizing the celiac plexus. The body of a radiotelemetry transmitter was placed in the abdominal cavity prior to closure of the abdomen. The catheter of the transmitter was tunneled subcutaneously to the medial thigh region where the tip was inserted into the abdominal aorta via the femoral artery.

13. Bilateral renal denervation (RDx)

Under inhalational general anesthesia, surgery was performed by a ventral midline laparotomy using aseptic technique. Bilateral RDx was performed using established methods in the rat (126). Briefly, the renal vessels were exposed and all visible nerves, fat and connective tissue removed. The renal vessels were

then painted with 10% phenol to destroy any remaining nerves. SHAM operation was performed by exposing and visualizing the renal vessels. The body of a radiotelemetry transmitter was then placed in the abdominal cavity prior to closure of the abdomen. The catheter of the transmitter was tunneled subcutaneously to the medial thigh region where the tip was inserted into the abdominal aorta via the left femoral artery.

14. Confirmation of regional denervation

On completion of the experimental period the rats were sacrificed with an intraperitoneal (IP) injection of pentobarbital (100 mg/kg). Liver, spleen, small intestine and both kidneys were collected from each animal, immediately frozen in liquid nitrogen and stored at -80°C for later analysis. The effect of CGx and bilateral RDx to successfully denervate their respective targets was assessed by measuring NE content of the tissue samples by reversed phase high performance liquid chromatography (HPLC) analysis with electrochemical detection. Results are reported as ng NE/gram of tissue. Dr. Veronika Mocko, Robert Burnett and James J. Galligan Jr. performed the tissue NE assay

15. Splanchnic blood flow and resistance

Non-hepatic splanchnic blood flow was approximated by directly measuring portal blood flow chronically in conscious rats using a transit-time ultrasound perivascular flow probe. This technique has previously been extensively validated (47). Under inhalational general anesthesia and via a ventral midline laporotomy, the abdominal viscera were reflected to the left and packed with

saline soaked gauze. The portal vein was isolated between the splenic vein and the bifurcation of the portal vein into the left and right portal trunks. A 2mm perivascular flow probe (2SB series, Transonic Systems, Inc, Ithaca, NY) was placed on the isolated section of the portal vein. The probe cable was passed through the abdominal wall and tunneled subcutaneously to exit the rat between the scapulae. The abdominal viscera were replaced and the abdominal incision was closed in two layers of interrupted sutures. An arterial catheter was implanted and tunneled subcutaneously to exit the rat between the scapulae into a stainless steel spring, along with the flow probe cable, attached to the rat by a loosely fitted rubber jacked. The rats were then loosely tethered in individual plastic cages to allow continuous access to the catheter and flow probe without handling or disturbing the animal.

Portal flow was recorded for approximately 60 minutes each day by connecting the flow probe to a dual channel flowmeter (T206, Transonic Systems, Inc), linked to a computerized data acquisition program (PowerLab). AP was measured simultaneously by connecting the arterial catheter to a pressure transducer which was linked to the computerized data acquisition program.

Portal blood flow (PF) approximates non-hepatic splanchnic blood flow, since under steady-state conditions the outflow from a vascular bed is equivalent to the inflow to that bed. Therefore splanchnic resistance (SR) was calculated as: SR = AP/PF.

16. Plasma catecholamine measurements

Blood sampling: 1 ml of blood was drawn from the arterial catheter into a 1 ml syringe primed with 25 µl (pH = 7) of an EGTA (9 mg/ml) and reduced glutathione (6 mg/ml) solution and placed on ice. The blood was centrifuged at 4°C (14,000 rpm for 15 minutes) and the plasma stored at -80°C until analysis.

NE concentrations: Catecholamines were determined in duplicate in100 µl of freshly thawed plasma by batch alumina extraction followed by reversed-phase high performance liquid chromatography separation with coulometric detection (HPLC-CD). The alumina extraction procedure and analyte quantification were performed using a method modified from the one originally reported by Holmes et al (118).

NE extraction: In a 1.5ml plastic tube, 100 µL freshly thawed plasma, 10 mg of acid washed alumina (EcoChrom MP Alumina A, MP Biomedicals, Germany), 15 µL of DHBA internal standard and 400 µL of 2M TRIS/0.5M EDTA buffer (pH 8.1) were added. After shaking for 25 min on a vortex mixer, the samples were briefly centrifuged and the supernatant discarded. The alumina pellet was then washed with D.I. water (18 MΩ), mixed for 15 s and then again centrifuged; this step was repeated twice. Catecholamines and metabolites were then eluted from alumina with 100 µL of 0.04 M phosphoric acid - 0.2 M acetic acid (20:80, v/v). The eluate was then directly injected onto the HPLC column (10 - 40 µL injection). The average extraction recovery of NE was 78.2%.

HPLC-CD: HPLC-CD was performed using a commercial system (ESA Biosciences, Inc, Chelmsford, MA) consisting of a solvent delivery module (model 584), an autosampler (model 542) cooled to 4°C and a Coulochem III detector which was equipped with a 5021A conditioning cell (electrode I) and a 5011A high sensitivity analytical cell (electrode II and III). Both cells use flow-through porous graphite electrodes. The high surface area of the detection electrodes results in an almost 100% reaction of the electroactive compound. Hydrodynamic voltammograms were obtained to determine the optimum potential for detection. The highest signal-to-noise results were obtained when electrode I was set at +200 mV, electrode II at +100 mV and electrode III at -280 mV. Chromatograms were obtained by monitoring the reduction current for working electrode III. The catecholamines and metabolites were separated on an HR-80 (C18, 3 µm particle size, 80 mm length x 4.6 mm I.D.) reversed-phase column (ESA Biosciences, Inc.). The mobile phase was a commercial Cat-A-Phase II (ESA Biosciences, Inc.) that consisted of a proprietary mixture of acetonitrile, methanol, phosphate buffer and an ion pairing agent (ca. pH 3.2). The optimum flow rate for the separation was 1.1 mL/min. The separation column was maintained at 35°C. The limit of detection of NE was 19.1 pg/ml.

17. Total body catecholamine kinetics

Total body NE clearance and spillover were determined by applying radioisotope dilution principles using established methods in the rat (136). To perform this

analysis, concentrations of ^{3}H-NE and NE were measured in arterial plasma after a 90 minute infusion of tracer amounts of ^{3}H-NE.

^{3}H-NE infusion: Levo-[ring-2,5,6-^{3}H]-NE (PerkinElmer, MA) was infused intravenously at 0.13 µCi/min/kg (288,888 dpm/min/kg) at a rate of 16 µl/min for 90 minutes. The infusion solution was prepared on ice immediately before administration as described by Keeton (136). Briefly a 10 ml solution was made first by adding 500 µl 0.2 mol/l acetic acid, 50 µl sodium sulfite (100 mg/ml) and 350 µl reduced glutathione (6mg/ml) to a conical polystyrene tube and mixing. The amount of ^{3}H-NE then added depended on the weight of the rats (~ 25 µl for 300 g rats), and 0.9% saline was used to bring the final volume of the solution to 10 ml. A syringe infusion pump was connected to the exteriorized venous catheter to deliver the solution to the rat intravenously.

Blood sampling: After infusion of ^{3}H-NE for 90 minutes, 1 ml of blood was drawn from the arterial catheter into a 1 ml syringe primed with 25 µl (pH = 7) of an EGTA (9 mg/ml) and reduced glutathione (6 mg/ml) solution and placed on ice. The blood was centrifuged at 4°C (14,000 rpm for 15 minutes) and the plasma stored at -80°C until analysis.

NE concentrations: Catecholamines were determined in duplicate in100 µl of freshly thawed plasma by batch alumina extraction followed by reversed-phase

high performance liquid chromatography separation with coulometric detection (HPLC-CD). (**14. plasma catecholamine measurements**)

^{3}H-NE concentrations: After HPLC analysis the NE fraction was collected using a Gilson model 203 fraction collector (Gilson Medical Electronics, Inc.) and the ^{3}H-NE was quantified using a Packard model TRI-CARB-2100TR liquid scintillation analyzer (Packard Instrument Co., IL, USA).

NE clearance and spillover calculations: Total body NE clearance and spillover were calculated using established methods (59, 136, 170).

NE clearance (ml/min) = ^{3}H-NE infusion rate (dpm/min) / steady state plasma ^{3}H-NE concentration (dpm/ml)

NE spillover (ng/min) = NE clearance (ml/min) x plasma NE concentration (ng/ml)

Both NE clearance and NE spillover were normalized to body weight and expressed as ml/min/kg and ng/min/kg respectively.

Plasma NE and ^{3}H-NE were measured by Robert Burnett and Martin Novotny, PhD.

18. Hind-limb blood flow and resistance

Hind-limb blood flow was chronically measured directly using a transit-time ultrasound perivascular flow probe placed on the terminal aorta, just proximal to the iliac bifurcation. Under inhalational general anesthesia and via a ventral midline laporotomy, the abdominal viscera were reflected and packed with saline soaked gauze. The terminal aorta was isolated from the vena cava and surrounding tissue using blunt dissection. A 2mm perivascular flow probe (2SB series, Transonic Systems, Inc) was placed on the isolated section of the aorta. The probe cable was passed through the abdominal wall and tunneled subcutaneously to exit the rat between the scapulae. The abdominal viscera were replaced and the abdominal incision was closed in two layers of interrupted sutures. An arterial catheter was implanted and tunneled subcutaneously to exit the rat between the scapulae into a stainless steel spring, along with the flow probe cable, attached to the rat by a loosely fitted rubber jacked. The rats were then loosely tethered in individual plastic cages to allow continuous access to the catheter and flow probe without handling or disturbing.

Hind-limb flow was recorded for approximately 60 minutes each day by connecting the flow probe to a dual channel flowmeter (T206, Transonic Systems, Inc), linked to a computerized data acquisition program (PowerLab). AP was measured simultaneously by connecting the arterial catheter to a pressure transducer which was linked to the computerized data acquisition program.

Hind-limb resistance (HLR) was calculated from hind-limb blood flow (HLF) and AP as: HLR = AP/HLF.

19. Hind-limb NE spillover

Hind-limb vascular bed NE spillover was measured in chronically instrumented, conscious, undisturbed rats by applying radioisotope dilution principle (59). This requires short-term infusion of ^{3}H-NE to achieve steady-state plasma concentrations, simultaneous sampling of arterial and venous blood from the hind-limbs and hind-limb blood flow measurements.

Instrumentation

Rats were chronically instrumented under inhalational anesthesia to allow measurements of hind-limb blood flow (**18. Hind-limb blood flow**) and catheters were placed in the terminal aorta (**5. Arterial catheterization**), terminal vena cava (**6. Venous catheterization: femoral**) and jugular vein (**7. Venous catheterization: jugular**) as described previously.

^{3}H-NE infusion: Levo-[ring-2,5,6-^{3}H]-norepinephrine (specific activity = 40-80 Ci/mmol, concentration 1 mCi/ml, PerkinElmer) was infused into the jugular vein as described previously (**17. Total body catecholamine kinetics**).

Blood sampling:

At the end of the 90 minute ^{3}H-NE infusion period a 1 ml arterial blood sample and 1 ml venous blood sample was obtained simultaneously from the aortic

catheter and vena cava catheter as described previously (**17. Total body catecholamine kinetics**). Hematocrit (Hct) was measured in duplicate from the arterial blood sample.

NE concentrations: NE was determined as previously described (**14. plasma catecholamine measurements**).

^{3}H-NE concentrations: 3H-NE concentration was determined as previously described (**17. Total body catecholamine kinetics**).

Hind-limb NE spillover calculations:

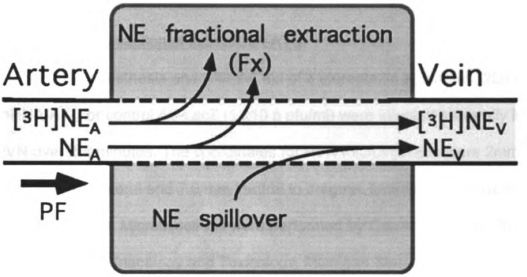
Calculation of hind-limb NE spillover were made using the established methods for radioisotope dilution estimation of regional NE spillover, published by Eisenhofer (59) and depicted in **figure 6**.

20. Downregulation of AT_1 receptors in the paraventricular nucleus

The method for silencing AT_{1a} receptors using an RNA interference (RNAi) strategy in the rodent brain has recently been described (37, 188).

Adenovirus preparation for AT1a RNAi

The DNA coding AT_{1a} short hairpin RNA (shRNA) has previously been synthesized and cloned into an adenoviral (Ad) vector (Ad-shRNA-AT_{1a}) (37). This construct was provided by Alex F. Chen, MD, PhD (Department of



NE spillover = $[(Fx \times NE_A) + (NE_V - NE_A)] \times PF$

where; PF = hind-limb blood flow x (1 – Hct), Fx = $(^3HNE_A - ^3HNE_V) / ^3HNE_A$

Figure 6: Radioisotope dilution estimates of regional norepinephrine (NE) spillover. Calculations are based on the product of organ plasma flow (PF) with the concentration of locally released NE into the venous drainage. A = artery, V = vein, Fx = fractional extraction of NE, Hct = hematocrit. From figure 5 Eisenhofer (59).

Pharmacology and Toxicology, Michigan State University). A standard Ad-LacZ virus was used as a control.

Bilateral PVN microinjection of Ad-shRNA-AT_{1a}

Under injectable anesthesia and with the aid of a stereotaxic apparatus, 200 ηl of Ad-shRNA-AT_{1a} or control Ad-LacZ (1 x10 p pfu/ml) were injected bilaterally into the PVN over 2-4 minutes. The coordinates for PVN microinjection were 2mm posterior, 1.7 mm lateral and 7.6 mm ventral to bregma. Microinjections were made at a 10^{o} angle. Microinjections were performed by Carrie Northcott, PhD (Department of Pharmacology and Toxicology, Michigan State University).

Verification of AT_{1a} Receptor Downregulation

Immediately following completion of the study the animals were sacrificed and decapitated. The brain was harvested and frozen immediately on dry ice. The levels of AT_{1a} receptor downregulation were determined by western analysis performed by Carrie Northcott, PhD (Department of Pharmacology and Toxicology, Michigan State University).

21. Animal euthanasia

At the completion of each study rats were euthanized by administration of an overdose of sodium pentobarbital (100 mg/kg). This is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

21. Statistical methods

These studies utilized a repeated measures approach by making multiple measurements in the same animal over time. Within group differences were assessed by a one-way repeated measures ANOVA with post-hoc multiple comparisons using Dunnett's procedure (GraphPad Instat 3). Between group differences were assessed by a two-way mixed design ANOVA and post-hoc testing at each time point performed using Bonferroni's procedure to correct for multiple comparisons (GraphPad Prism 4). A p-value of < 0.05 was considered significant. When only two groups were compared, Student's t-test was used. All results are presented as mean ± SE.

CHAPTER TWO: THE HYPERTENSIVE RESPONSE TO CHRONIC LOW-DOSE ANGIOTENSIN II IS DEPENDENT ON ARTERIAL PRESSURE MEASUREMENT METHOD AND SALT INTAKE

It has been reported that when arterial pressure (AP) is measured by radiotelemetry, chronic low-dose AngII infusion enhances pressor responses to external stimuli but does not cause sustained hypertension (206). This finding has important implications given that historically most investigations into chronic low-dose AngII hypertension have been performed whereby AP is measured using the tail cuff method or by exteriorized arterial catheter. AP measurements by tail-cuff and radiotelemetry have been directly compared in chronic AngII hypertension (206); however the effect of chronic catheterization is unclear. The purpose of this study was to compare the hypertensive response to low-dose AngII in rats chronically instrumented with exteriorized arterial catheters to rats implanted with radiotelemetry transmitters.

A recent statement from the American Heart Association's Council on High Blood Pressure Research comprehensively reviewed current recommendations for AP measurement methods in experimental animals (152). Selection of AP measurement technique should be driven by the experimental objective, however, direct methods such as radiotelemetry and exteriorized catheters are generally preferred as they assess the dynamic nature of AP (152). Radiotelemetry and exteriorized catheters both provide accurate, reliable and extensively validated direct measurements of AP. Although the specific advantages and disadvantages of the two methods are somewhat distinct, their

ability to accurately and dynamically measure AP make their application almost interchangeable (152). Exteriorized catheters and tethering systems allow for measurements to be made in conscious and relatively undisturbed animals. However, it has been proposed that tethering may cause some degree of stress to the animals. It is unclear what influence this may have on AP in AngII hypertension, a very commonly studied experimental model.

One hallmark of chronic low-dose AngII hypertension is that the elevation in AP is salt-sensitive. We tested the hypothesis that low-dose AngII infusion will cause a salt dependent increase in AP irrespective of AP measurement method, but the magnitude of the AP increase will be greater in tethered rats, due to the stress of tethering. In addition we hypothesized that simulation of tether stress on rats with radiotelemetry transmitters will exacerbate the hypertension. Determining the effect of AP measurement technique on the response to AngII was particularly critical for the work in thesis as some studies utilized radiotelemetry, whereas other studies demanded the use of exteriorized catheters.

Methods

Experimental Protocols

After dietary acclimatization to a 0.4% or 2% NaCl diet for 7 days, rats were instrumented with an exteriorized arterial catheter or radiotelemetry transmitter to monitor AP and heart rate (HR). Following 7 days of recovery and a 7 day control period, an AngII or physiological saline filled osmotic minipump was implanted subcutaneously, to deliver AngII (150ng/kg/min) or vehicle for 14 days. Rats were

allowed free access to 0.4% or 2% NaCl diet and distilled water for the duration of the experiment. In addition, a separate group of rats fed 2% NaCl were implanted with radiotelemetry devices and fitted with a rubber jacket and tethered. These rats were subjected to the same protocol as above except for a 1 hour simulation of tether handling on control day 7 and AngII infusion days 7 and 14. This simulation involved manipulating the tether system to mimic AP measurement by exteriorized catheter in these rats.

Animals

A total of 53 rats were studied. Initially, the groups studied on a high salt diet (2% NaCl) were radiotelemetry vehicle (HRV, n=4), catheter vehicle (HCV, n=4) radiotelemetry AngII (HRA, n=8) and catheter AngII (HCA, n=8). On a normal salt diet (0.4% NaCl) the groups studied were radiotelemetry vehicle (NRV, n=4), catheter vehicle (NCV, n=4) radiotelemetry AngII (NRA, n=7) and catheter AngII (NCA, n=8). Then to assess the effect of tethering on the response to AngII in rats fed 2% NaCl and instrumented with radiotelemetry transmitters, 3 standard radiotelemetry rats were compared to 3 rats with implanted transmitters that were fitted with a rubber jacket and tethered.

Results

The mean arterial pressure (MAP) response to chronic infusion of AngII or vehicle is shown in **figure 7**. During the control period MAP was indistinguishable between the groups irrespective of AP measurement method and independent of salt diet. In rats fed 2% NaCl the average MAP for the 7 day control period was

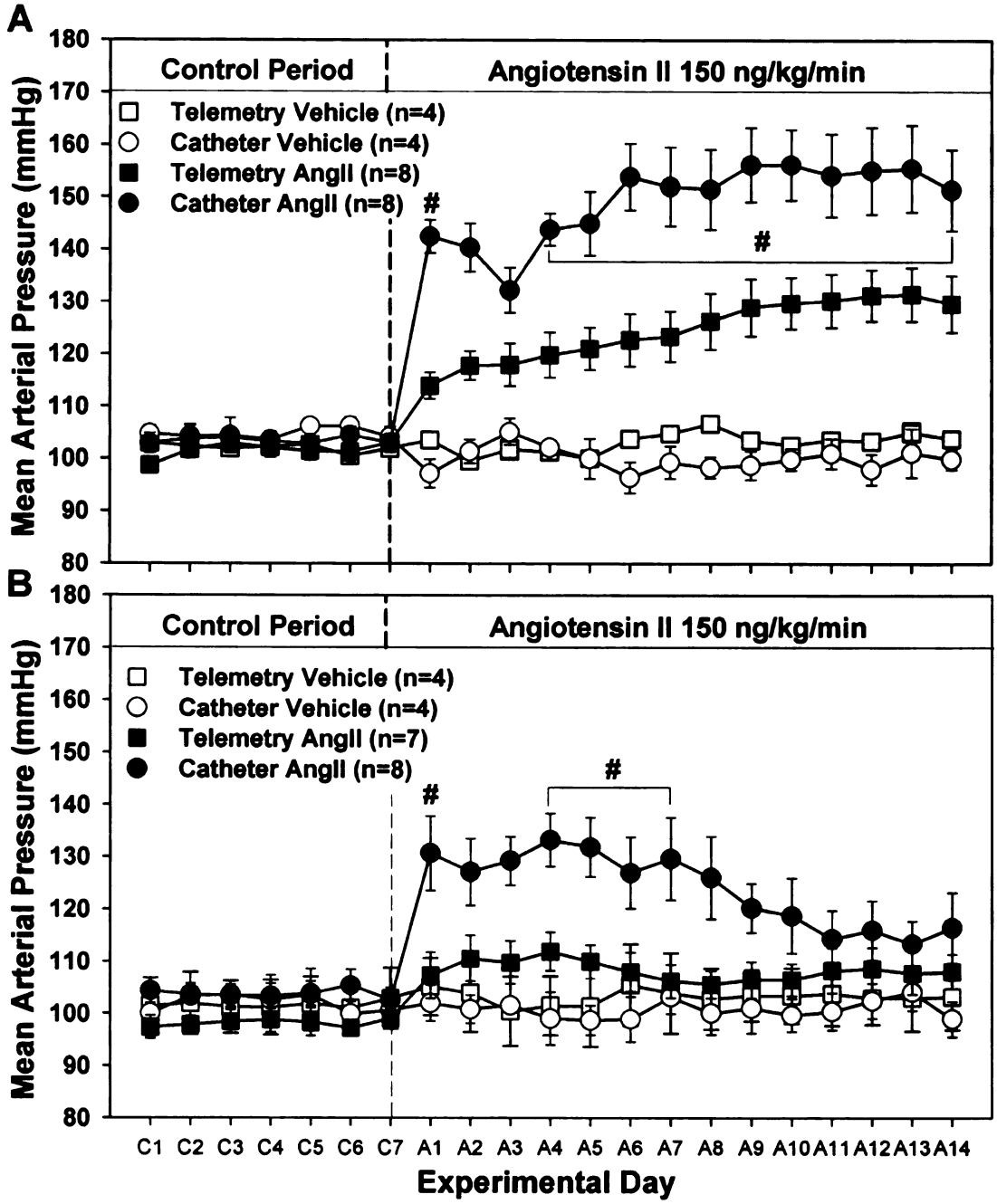


Figure 7: Mean arterial pressure (MAP) measured by radiotelemetry and arterial catheter in the standard AngII infusion protocol. Rats were fed 2% NaCl **(A)** or 0.4% NaCl **(B)** diet. # = $p < 0.05$ compared to radiotelemetry rats infused with AngII.

very similar among all groups (HRV 102 ± 1, HCV 104 ± 1, HRA 102 ± 1 and HCA 103 ± 1 mmHg). Likewise the average MAP for the 7 day control period was very similar among all groups fed 0.4% NaCl (NRV 102 ± 5, NCV 102 ± 3, NRA 98 ± 2 and NCA 104 ± 2 mmHg). Additionally the MAP measurements during vehicle infusion were indistinguishable between groups, irrespective of AP measurement technique and independent of salt diet. MAP was similar in all vehicle treated groups on day 14 of vehicle infusion (HRV 104 ± 1, HCV 100 ± 2, NRV 103 ± 6 and NCV 99 ± 3 mmHg).

A salt-sensitive and sustained increase in AP occurred in response to infusion of AngII irrespective of measurement method. However the magnitude of the hypertensive response to AngII was dependent on the AP measurement method and was markedly increased in catheterized rats compared to radiotelemetry rats. This was particularly evident in the rats fed 2% NaCl diet where the MAP response to AngII was significantly greater in catheterized rats on day 1 of AngII infusion (HRA 114 ± 3 and HCA 142 ± 3 mmHg, $p<0.05$). The pressor response remained significantly greater in tethered rats from day 4 to 14 of AngII infusion (HRA 130 ± 5 and HCA 151 ± 8 mmHg on day 14 AngII infusion, $p<0.05$).

The hypertensive response to AngII was also greater in rats fed 0.4% NaCl when AP was measured by externalized catheters, particularly during the first week. However, this difference was only significant on AngII infusion days 1 (NRA 107 ± 4 and NCA 131 ± 7 mmHg, $p<0.05$), and 4 to 7. Although catheterized rats still

had a higher MAP on AngII infusion day 14 the difference was not statistically significant (NRA 108 ± 3 and NCA 117 ± 7 mmHg).

The heart rate (HR) response to chronic infusion of AngII or vehicle is shown in **figure 8**. Although catheterized rats tended to have a lower HR than rats instrumented with radiotelemetry transmitters, both during the control and infusion periods, these differences were not statistically significant.

The MAP response to AngII in standard telemetry rats and telemetry rats fitted with a tether is shown in **figure 9**. During the control period tethering did not affect MAP (106 ± 1 versus 105 ± 1 mmHg). Tethering the rats tended to increase the hypertensive response to AngII, however this was not statistically significant (114 ± 8 versus 129 ± 9 mmHg, AngII infusion day 14). The effect of simulating tether handling on MAP in standard telemetry rats and telemetry rats fitted with a tether is shown in **figure 10**. Simulation of tether handling had no effect on MAP during the control period. However, mimicking the conditions of tethering enhanced the pressor response to AngII in telemetry rats fitted with a tether on both day 7 (151 ± 3 mmHg) and 14 (139 ±2 mmHg) of AngII infusion, but had little effect on standard radiotelemetry rats on either day 7 (123 ± 3 mmHg) or 14 (121 ± 3 mmHg) of AngII infusion.

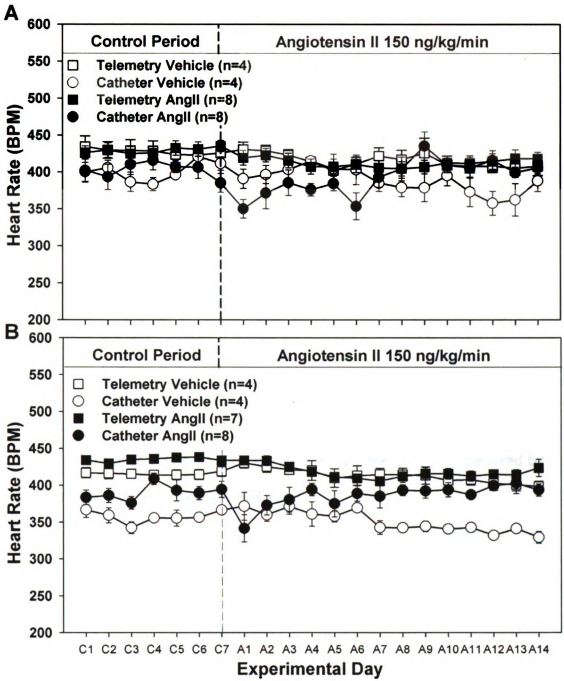


Figure 8: Heart rate (HR) measured by radiotelemetry and arterial catheter in the standard AngII infusion protocol. Rats were fed 2% NaCl **(A)** or 0.4% NaCl **(B)** diet.

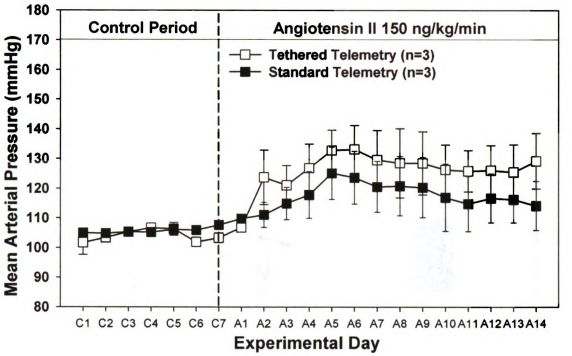


Figure 9: The effect of tethering on the MAP response to AngII measured by radiotelemetry. Rats were fed 2% NaCl diet.

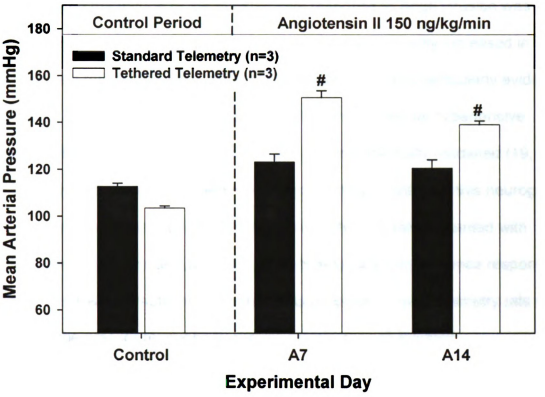


Figure 10: The effect of simulating tethering stress on MAP measured by radiotelemetry. MAP measured by radiotelemetry under standard or tethered conditions during 1 hour simulation of tethering stress on control day 7 and AngII infusion days 7 (A7) and 14 (A14) in rats fed 2% NaCl diet. # = $p < 0.05$ compared to standard telemetry.

Discussion

This study shows that chronic infusion of low-dose AngII results in a sustained and salt-sensitive increase in AP, irrespective of AP measurement method. However, the magnitude of the hypertensive response to AngII infusion was dependent on the AP measurement method and was markedly increased in catheterized rats compared to radiotelemetry rats. This was particularly evident in rats fed a high salt diet. Evidence suggests that the additional hypertensive response to AngII in animals fed a high salt is sympathetically mediated (19, 139). Therefore, the effect of tethering may be acting to enhance this neurogenic mechanism in rats fed a 2% NaCl diet. Finally, tethering rats implanted with radiotelemetry transmitters was not sufficient to significantly enhance responses to AngII. However simulating tether handling in tethered, radiotelemetry rats did enhance the pressor response to chronic low-dose AngII infusion.

Interestingly AP was indistinguishable between groups, irrespective of AP measurement technique, during the control period and throughout the entire vehicle infusion. This indicates good agreement between the two measurement methods during baseline conditions and suggests that the stress of catheterizing normotensive rats does not itself influence AP. However, it appears as though catheterization and tethering sensitizes the rats to the hypertensive actions of AngII. The effect of tethering to increase the magnitude of the response to AngII is evident immediately as indicated by significantly greater increases in AP on day 1 of AngII infusion. It is unclear whether this effect of tethering is specific to

AngII or may be indicative of a more widespread sensitization to hypertensive stimuli. The findings of this study are consistent with others demonstrating that cardiovascular responses to AngII are influenced by the measurement method (121, 206).

Most recent studies investigating blood pressure measurement methods have focused on comparing invasive and non-invasive methodologies (114, 121, 128, 149, 276) and less attention has been paid to comparing the direct methods to each other. Direct methods are accurate and reliable, and allow chronic and dynamic long term recordings in conscious undisturbed animals (148, 152, 264). As a result they are considered the standard for arterial pressure measurement in experimental studies (148, 152, 264). This study emphasizes the importance of considering how AP measurement method may influence cardiovascular responses. Many environmental influences such as ambient temperature (35, 285) are well documented to influence AP and metabolic rate, and therefore it is not surprising that stresses involved in measuring AP also influence pressor responses.

Although the mechanism by which the stress of tethering enhances AP responses to AngII in this study is unclear, extensive evidence implicates AngII in mediating many of the responses to stress. For example, activation of central AT1 receptors increases release of hypothalamic and adrenal stress hormones and inhibition of AT1 receptors reduces the stress sensitivity of the hypothalamo-

pituitary-adrenal axis in spontaneously hypertensive rats and responses to isolation stress (5, 213). Saavedra has published extensively on the relationship between AngII and stress responses, and this was recently summarized in a comprehensive manner (224). Perhaps the most intriguing finding is that brain AT1 receptor expression is significantly increased during stress (224). This provides a plausible explanation for the enhanced AP response to AngII in tethered animals; the stress of tethering may increase central AT1 receptor expression and result in increased sensitivity to exogenous AngII. However this hypothesis needs to be specifically tested.

Perspectives

Contrary to previous reports, chronic infusion of low-dose AngII does cause a salt-sensitive and sustained elevation in AP (206) and therefore provides a valuable tool to study neurogenic mechanisms of hypertension. However, the magnitude of the hypertensive response to infusion of chronic low dose AngII is dependent on blood pressure measurement method. Therefore the measurement technique needs to be considered when interpreting AP responses.

CHAPTER THREE

King AJ and Fink GD. Chronic low-dose angiotensin II infusion increases venomotor tone by neurogenic mechanisms. *Hypertension* 48: 927-933, 2006.

Emerging evidence suggests that sympathetic nervous system (SNS) activation may represent a common neurogenic mechanism of hypertension in both human essential hypertension (3, 65, 72, 81, 98, 233) and many experimental animal models (6, 24, 156, 251). Angiotensin II (AngII) has been identified as a humoral factor implicated in activating the SNS in human hypertension (12, 99); and the pressor response to infusion of chronic low-dose AngII in animals has been shown, at least in part, to be sympathetically driven (20, 97, 231). Advances have been made in the elucidation of the central pathways involved in mediating this sympathoexcitatory effect, suggesting that systemically delivered AngII likely activates critical circumventricular organs (21, 43, 82, 144) with efferent projections to brain centers known to influence SNS activity (144). Oxidative stress may mediate this central sympathoexcitatory effect (30). However, there is still substantial uncertainty as to the critical peripheral target and the hemodynamic response to this increased sympathetic activity.

In this study we investigated the possibility that the venous circulation may be an important target for AngII induced SNS activation. The venous system contains approximately 70% of the blood volume (201), mostly in the small veins and venules, and has been shown to be more sensitive to sympathetic activation than arterioles (119). In particular the splanchnic venous bed is densely innervated by the sympathetic nervous system and represents the most important active capacitance bed in the body (100, 102, 222). It has been shown that increases in

splanchnic SNS activity causes a translocation of blood towards the heart, increasing cardiac diastolic filling and cardiac output (101, 102).

It is well established that chronic low-dose AngII infusion is a salt-sensitive model of hypertension, and there is evidence to suggest that the additional hypertensive effect of salt is mediated by SNS activation (18, 19, 194, 231, 259). While the most compelling evidence for SNS activation in response to salt is described in rats (18, 19, 194, 231, 259), it has also recently been demonstrated in rabbits (180) using repeated assessment of response to ganglion blockade. More importantly there is considerable evidence that neurogenic mechanisms may play a role in the pathophysiology of salt sensitivity in human essential hypertension (26, 28, 29, 91, 146, 186, 240). A recent study measuring spontaneous arterial baroreflex sensitivity convincingly demonstrated abnormalities in autonomic control of the cardiovascular system in association with salt-sensitivity, supporting the hypothesis that salt sensitivity is at least in part neurogenically driven (44). In this study we test the hypothesis that chronic infusion of low-dose AngII increases venous smooth muscle tone by activation of the SNS, in a salt-sensitive manner.

Mean circulatory filling pressure (MCFP) is the pressure measured in the vasculature immediately following cardiac arrest, after pressures in all parts of the circulation are made to equilibrate, and represents the effective driving force for venous return to the heart (110). The major determinants of MCFP are

compliance of the venous system and blood volume (288), and MCFP is considered the best methodology for determination of body venous tone (201). We used repeated measures of MCFP in conscious, undisturbed rats fed a normal (0.4%) or high (2%) NaCl diet to investigate venomotor tone changes in chronic low-dose AngII induced hypertension.

Methods

Experimental Protocol

Rats were acclimatized to a 0.4% or 2% NaCl diet for 7 days and then chronically instrumented to allow repeated measures of MCFP in conscious, undisturbed animals. Following 4 days of recovery and a 3 day control period, an AngII or physiological saline filled osmotic minipump was implanted subcutaneously, to deliver AngII (150ng/kg/min) or vehicle control for 14 days. AP was measured at the same time daily for the duration of the experiment and recorded as a 10 minute average. MCFP was measured in duplicate before and starting 5 minutes after acute ganglion blockade with hexamethonium (30 mg/kg IV) on control day 2 and AngII infusion days 1, 3, 7 and 14. The duplicate measure of MCFP was always 10 minutes after the previous one. Prior to the MCFP measurements the catheters were flushed and connected to pressure transducers. Rats were then allowed to sit undisturbed for 20 minutes. Hct and PV were also measured on these days, at least 6 hours after completion of the MCFP measurements. During the entire experimental protocol rats were allowed free access to either 0.4% NaCl or 2% NaCl diet and distilled water.

Animals

A total of 24 rats were studied in 4 different groups; 0.4% NaCl diet + vehicle (NV, n=4), 0.4% NaCl diet + AngII (NA, n=8), 2% NaCl diet + vehicle (HV, n=4) and 2% NaCl diet + AngII (HA, n=8).

Results

The mean arterial pressure (MAP) and heart rate (HR) response to chronic subcutaneous infusion of AngII (150 ng/kg/min) or saline vehicle is shown in **figure 11**. MAP was not different between the 4 groups during the control period (NV 101 ± 3, NA 100 ± 2, HV 99 ± 3, and HA 101 ± 2 mmHg). Similarly HR was not different between the 4 groups during the control period (NV 437 ± 7, NA 413 ± 12, HV 394 ± 25 and HA 416 ± 8 BPM). Additionally MAP (NV 101 ± 1 versus HV 100 ± 2 mmHg on day 14 of vehicle infusion) and HR (NV 391 ± 12 versus HV 391 ± 14 BPM on day 14 of vehicle infusion) did not change in response to vehicle infusion. AngII caused a significant increase in MAP for the entire duration of infusion, irrespective of salt diet, however the magnitude of the increase was much greater in rats on a 2% NaCl diet (NA 117 ± 3 versus HA 155 ± 7 mmHg on day 14 of AngII infusion). HR was significantly decreased on AngII infusion days 3 to 6 in rats on a 2% NaCl diet, but was no different from rats fed a 0.4% NaCl diet for the remainder of the infusion period.

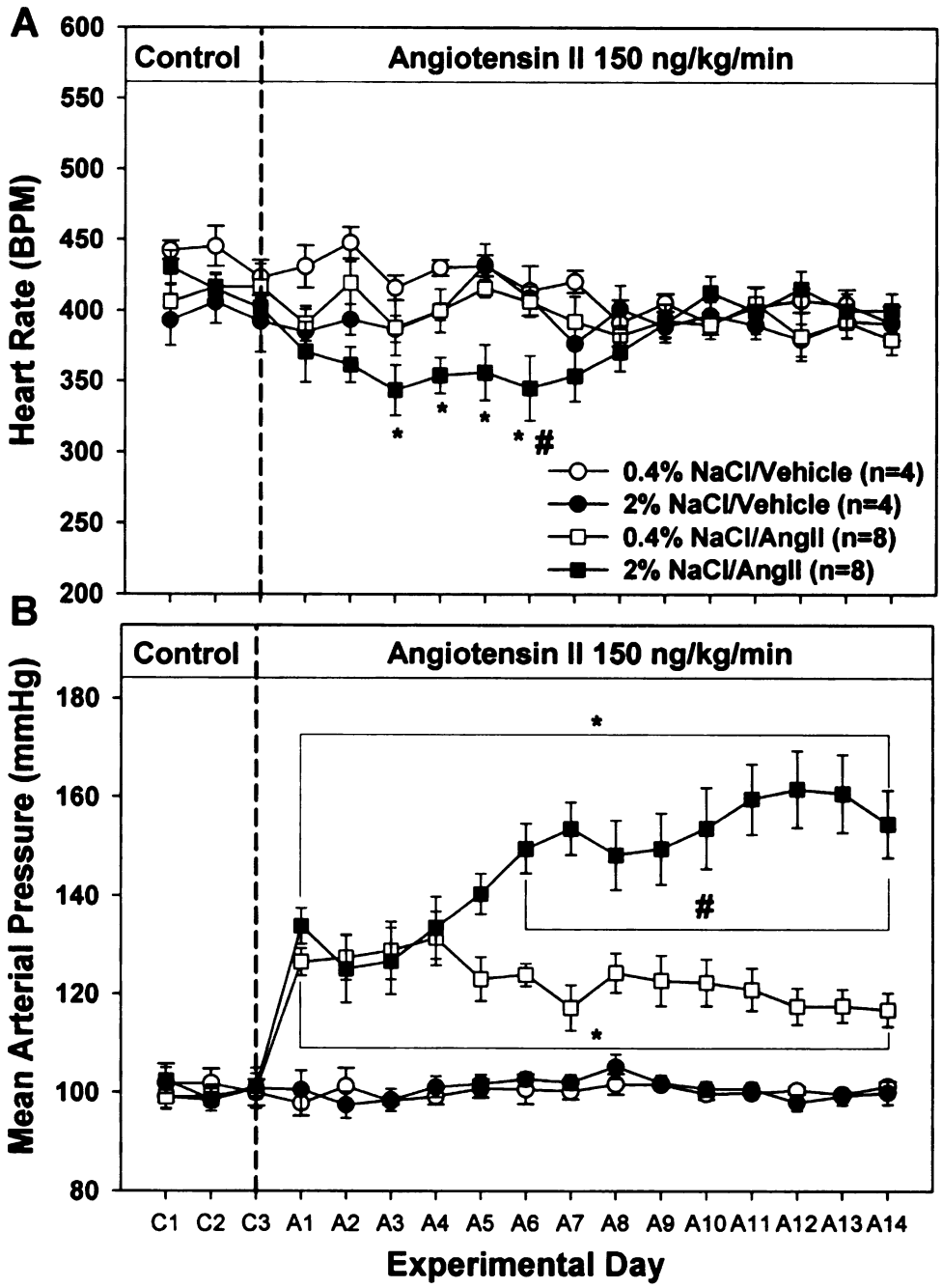


Figure 11: HR and MAP in animals catheterized for mean circulatory filling pressure (MCFP) measurements. HR **(A)** and MAP **(B)** response to infusion of AngII or vehicle in rats fed 2% or 0.4% NaCl diet. * = $p < 0.01$ compared to control period. # = $p < 0.01$ compared to 0.4% NaCl.

Figure 12 shows BV and MCFP responses to chronic subcutaneous infusion of AngII or saline vehicle, measured on control day 2 and infusion days 1, 3, 7 and 14. BV tended to be higher during the control period in rats fed a 0.4% NaCl diet (NV 27 ± 1 and NA 26 ± 1 ml) compared to rats on a 2% NaCl diet (HV 25 ± 1 and HA 24 ± 1 ml), however this was not statistically significant. Also there were no statistically significant changes in BV in response to vehicle or AngII infusion in any group. MCFP tended to be higher during the control period in rats fed a 0.4% NaCl diet (NV 7.3 ± 0.7 and NA 7.1 ± 0.4 mmHg) compared to rats on a 2% NaCl diet (HV 6.5 ± 0.2 and HA 6.4 ± 0.3 mmHg), however this was not statistically significant. There were no statistically significant changes in MCFP in response to vehicle infusion in rats fed either diet (NV 7.0 ± 0.4 and HV 6.6 ± 0.2 mmHg on day 14 of infusion) or in response to AngII infusion in rats fed 0.4% NaCl diet (NA 6.9 ± 0.3 mmHg on day 14 of infusion). However there was a significant and marked increase in MCFP in response to AngII infusion in rats fed a 2% NaCl diet. This increase was significant on day 1 (8.5 ± 0.3 mmHg) of AngII infusion, and MCFP was further increased on days 3 (8.5 ± 0.8 mmHg), 7 (9.8 ± 0.7 mmHg) and 14 (9.8 ± 0.8 mmHg) of AngII infusion.

Peak MAP response to acute ganglion blockade with hexamethonium (30 mg/kg IV) is shown in **figure 13** along with the MCFP response 5 and 15 minutes after hexamethonium administration. MAP response to hexamethonium was no different between the 4 groups during the control period (NV -47 ± 3, NA -41 ± 2, HV -41 ± 4, and HA -41 ± 2 mmHg) and did not change in response to vehicle

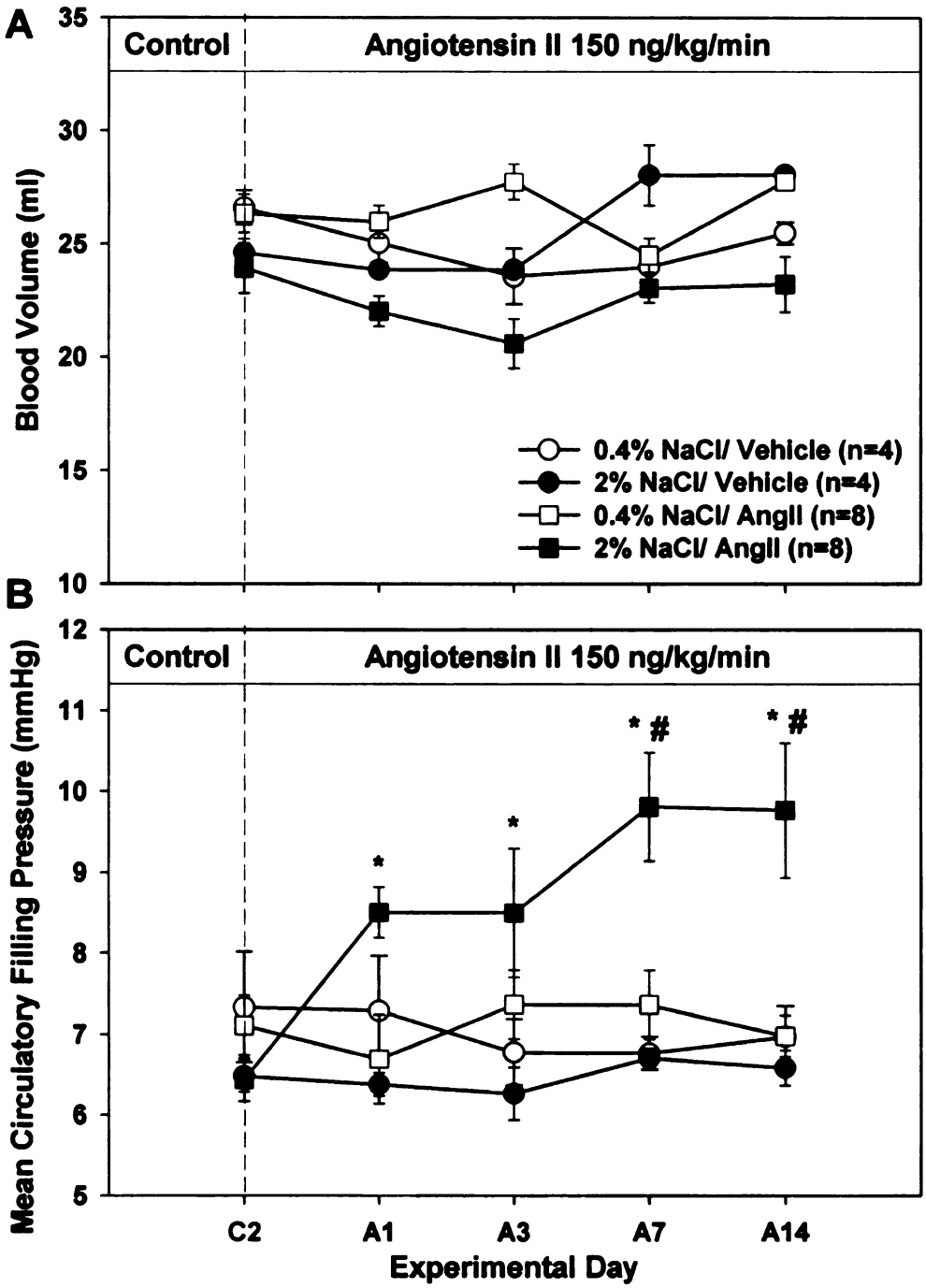


Figure 12: Blood volume and MCFP responses in the standard AngII infusion protocol. The response of blood volume **(A)** and MCFP **(B)** to infusion of AngII or vehicle started in rats fed 2% or 0.4% NaCl diet. * = $p < 0.01$ compared to control period. # = $p < 0.01$ compared to 0.4% NaCl.

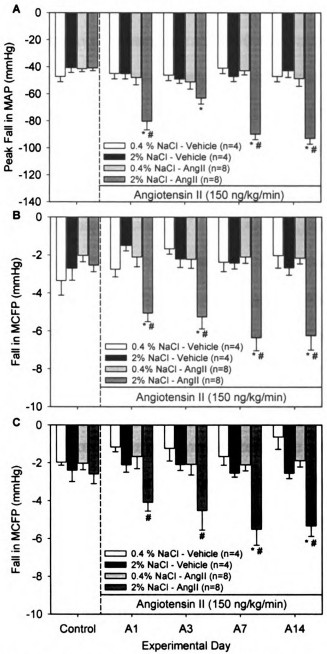


Figure 13: Hemodynamic response to acute ganglion blockade in the standard AngII infusion protocol. Peak fall in mean arterial pressure (MAP) **(A)** and decline in mean circulatory filling pressure (MCFP) measured 5 minutes **(B)** and 15 minutes **(C)** after hexamethonium. * = $p < 0.01$ compared to control period. # = $p < 0.01$ compared to 0.4% NaCl.

infusion in rats fed either diet (NV -47 ± 4 and HV -43 ± 5 mmHg on day 14 of infusion) or in response to AngII infusion in rats fed 0.4% NaCl diet (NA -49 ± 6 mmHg on day 14 of infusion). However there was a significant and marked increase in MAP response to hexamethonium, in response to AngII infusion, in rats fed a 2% NaCl diet. This increase was significant on day 1 (-80 ± 6 mmHg) of AngII infusion, remained statistically significantly increased on day 3 (-63 ± 4 mmHg) and was further increased on days 7 (-90 ± 4 mmHg) and 14 (-93 ± 4 mmHg) of AngII infusion. MCFP response 5 minutes after hexamethonium was no different between the 4 groups during the control period (NV -3.3 ± 0.8, NA -2 ± 0.3, HV -2.7 ± 0.6, and HA -2.5 ± 0.3 mmHg) and did not change in response to vehicle infusion in rats fed either diet (NV -2 ± 0.7 and HV -2.7 ± 0.4 mmHg on day 14 of infusion) or in response to AngII infusion in rats fed 0.4% NaCl diet (NA -2.2 ± 0.3 mmHg on day 14 of infusion). However there was a significant and marked increase in MCFP response to hexamethonium in AngII infused rats fed a 2% NaCl diet. This increase was significant on day 1 (-5 ± 0.4 mmHg) of AngII infusion, remained statistically significantly increased on day 3 (-5.3 ± 0.6 mmHg) and was further increased on days 7 (-6.4 ± 0.7 mmHg) and 14 (-6.2 ± 0.8 mmHg) of AngII infusion. MCFP response 15 minutes after hexamethonium was very similar to 5 minutes (**figure 13**).

Discussion

Consistent with previous studies, we have demonstrated a salt-sensitive hypertension in response to infusion of chronic low-dose AngII. This dose of

AngII, when administered subcutaneously via osmotic minipump, has been shown to increase plasma AngII levels approximately 2 fold and result in plasma concentrations within the pathophysiological range (267). More importantly our results support the conclusion (18, 19, 194, 231, 259) that the additional hypertensive response to salt is sympathetically driven, indicated by increased AP responses to acute ganglion blockade. AP response to ganglion blockade did not change during AngII infusion in rats fed a normal salt diet. This indicates that the rise in AP to AngII in rats on normal salt diet may not be neurally mediated. However the expected response to increased AP is baroreflex mediated sympathoinhibition. The finding that the AP response to ganglion blockade did not decrease in this group suggests that AngII may prevent this sympathoinhibition. This is consistent with a previous report which found plasma norepinephrine levels were unchanged by AngII alone, but markedly increased when administered in combination with a high salt diet (231).

The major new finding in this study was that MCFP was increased in response to AngII infusion, beginning on day 1 and increasing further on days 3, 7 and 14, only when administered in combination with high dietary salt. In the absence of a change in blood volume, increases in MCFP represent an increase in venomotor tone (83, 201, 288). In the present study blood volume was unchanged. Therefore we have demonstrated the ability of AngII, in combination with high dietary salt, to chronically increase venous smooth muscle tone. This increase in MCFP was essentially abolished by acute ganglion blockade with

hexamethonium suggesting that the increase in venomotor tone was neurogenically driven.

Interestingly the SNS activation demonstrated in the AngII infused group fed a high salt diet appeared to be regionally heterogeneous. Venous sympathetic activity clearly increased, as indicated by a hexamethonium sensitive increase in MCFP; however this occurred in the absence of any evidence of increased cardiac sympathetic activity. HR can provide a useful index of cardiac sympathetic activity (135) and no tachycardia was observed over the 14 day infusion period. The finding of regionalized sympathetic activation is consistent with other experimental findings demonstrating differential control of lumbar and renal sympathetic nerve activity in rabbits (215). Furthermore, by using regional norepinephrine spillover techniques, Esler and colleagues have elucidated the importance of regionalized sympathetic activation in human cardiovascular disease (2, 62, 67, 68, 70). However the apparent regionalized sympathetic activation seen in this study may be due to the limitation of using HR as an index of sympathetic nerve activity or may be a result of temporal differences in the activation pattern.

Reduced vascular capacitance has been documented as a feature of human essential hypertension (225), and in many experimental animal models including DOCA-salt hypertension(83), spontaneously hypertensive rat (175, 261), the angiotensin-dependent two-kidney, one clip hypertensive rat (287) and in one-

kidney, one-clip Goldblatt hypertension (289). The increase in venous constriction and subsequent decrease in whole-body venous capacity is commonly neurogenically driven in these models (83, 175) and blood volume tends to be normal or decreased (261, 287, 289). Also it has recently been shown that the salt-sensitivity of DOCA salt hypertension is not mediated primarily by volume related mechanisms, but rather an increased sensitivity of blood pressure to total body water content (258). This again emphasizes the importance of reduced vascular capacitance in salt-sensitive hypertension. Vascular capacitance is strongly controlled by the SNS and is predominately influenced by the compliance of the venous system, suggesting that sympathetically driven increases in venomotor tone may be an important hemodynamic mechanism of hypertension.

It has previously been shown that acute infusion of AngII increases venous tone in rats by both direct and neurogenic mechanisms (250). Also, chronic infusion of AngII, in dogs fed a high salt diet, increased MCFP and resulted in an increase in total peripheral resistance and a decrease in cardiac output (292). This marked increase in MCFP occurred in the absence of blood volume changes, indicating a reduction in vascular compliance mediated by venoconstriction. However, the salt-sensitivity, temporal profile, and the contribution of SNS activation to venomotor tone changes in this well characterized model of chronic low-dose AngII infusion in rats has not been investigated.

We propose that increases in venomotor tone, particularly in the active capacitance bed of the splanchnic circulation, contribute to AngII-salt hypertension by increasing the driving force for venous return to the heart resulting in a translocation of venous blood to the less compliant arterial system. Guyton's group have suggested that increases in MCFP will result in chronic hypertension only when associated with a simultaneous impairment of renal excretory function (106, 108, 172). It has been well documented by this same group that AngII is one factor that can markedly impair renal excretory function (291). Indeed the present study is consistent with this, as venous capacitance was greatly reduced with no change in total measured blood volume, suggesting a venous to arterial translocation of blood. The failure of this arterial translocation to elicit a net sodium and water loss indicates the expected impairment in renal excretory function. When it has been assessed, MCFP has been found to be elevated in virtually all experimental models of hypertension, in multiple species, making it difficult to confirm a causal or merely associative role in hypertension (78, 83, 172, 175, 218, 219, 261, 287, 289) . Therefore it is important to note that MCFP was unchanged in the AngII infused group fed a normal salt diet. Considering these findings together, it is likely that the increases in MCFP seen in the AngII-salt group contribute to the genesis of the hypertension in this model.

Perspectives

What is the physiological impact of reduced vascular capacitance in hypertension? Blood volume generally is not increased, so reduced vascular

capacitance accounts for the increased "effective blood volume" characteristic of established hypertension. That is, hypertensive individuals behave as if they are volume expanded: for example, they exhibit greater increments in cardiac output and natriuresis to acute volume loads, and larger hypotensive responses to diuretic drug treatment. Reduced vascular capacitance therefore makes a significant contribution to the circulatory physiology of hypertension. The SNS, by virtue of its influence on splanchnic venous smooth muscle tone, is the principal factor regulating vascular capacitance. A better understanding of sympathetic control of capacitance vessels, especially in the setting of excess salt intake, could provide new approaches to the treatment or prevention of high blood pressure.

CHAPTER FOUR

King AJ, Osborn JW and Fink GD. Splanchnic circulation is a critical neural target in angiotensin II – salt hypertension in rats. *Hypertension* 50: 547-556, 2007.

We have recently shown, using repeated measures of mean circulatory filling pressure (MCFP) in conscious undisturbed rats, that chronic infusion of angiotensin II (AngII), only when administered in combination with a high salt diet, activates the sympathetic nervous system (SNS) to increase venomotor tone (140). This increase in venomotor tone may contribute to the pathogenesis of AngII-salt hypertension, by increasing central blood volume, resulting in a translocation of blood from the highly compliant venous system to the less compliant arterial circulation (140). This redistribution of blood volume, along with the well documented impairment of renal excretory function caused by AngII (291) would be major factors in increasing arterial pressure (AP) in this model (106, 108, 172).

Splanchnic veins and venules account for most of the active capacitance responses in the circulation, and are richly innervated by the SNS (100, 102, 222). In fact it has been estimated that innervation to the non-hepatic splanchnic organs accounts for half of the total norepinephrine (NE) released in the entire body (4) . Therefore our recent observations in AngII-salt hypertension of neurogenically mediated increases in whole body venous tone, would be best explained by increased SNS activity to the splanchnic circulation (140). Indeed this is consistent with the previously reported finding of significant increases in splanchnic nerve activity, as assessed by direct nerve recordings, in conscious rats during chronic AngII infusion (167). Together these findings indicate the splanchnic circulation may be an important peripheral target for AngII mediated

sympathoactivation in experimental hypertension. Vascular resistance increases in the hepatosplanchnic circulation before any other bed in humans with borderline hypertension (248) . Therefore increased sympathetic activity to the splanchnic circulation may represent a common stage in the development of hypertension.

The purpose of this study was to investigate the role of sympathetic nerve activity to the splanchnic circulation in the pathogenesis of AngII hypertension in the rat. In particular, the importance of the splanchnic SNS to increases in arterial pressure (AP) and whole body venous tone in AngII-salt hypertension were assessed. Approximately 95% of sympathetic postganglionic neurons innervating this vascular bed in the rat have their cell bodies in the celiac and superior mesenteric ganglia (120, 212, 262). These two ganglia are fused in the rat and are commonly referred to as the celiac or solar plexus (39) . We used surgical ablation of this plexus (celiac ganglionectomy (CGx)) to investigate our hypothesis. Specifically we tested the hypothesis that CGx will attenuate increases in whole body venous tone and AP during AngII infusion only in rats fed a high salt diet. We previously demonstrated neurogenically mediated increases in whole body venous tone in response to AngII only in the setting of high dietary salt intake (140). Importantly, approximately 70% of renal post ganglionic neurons are localized to the paravertebral chain ganglia (39, 77, 245) in rats and therefore should be spared by CGx. However, the importance of the renal nerves in various experimental models of hypertension, including AngII

hypertension, has previously been reported (127, 132, 160, 187, 192, 286). Therefore to thoroughly assess the possibility that CGx was exerting its effects via renal denervation, separate groups of rats received selective bilateral renal denervation (RDx).

The effect of CGx and RDx on chronic AngII hypertension was initially determined by instrumenting rats with radiotelemetry transmitters to allow remote monitoring of AP. We then used repeated measurements of MCFP in conscious, undisturbed rats to investigate the effect of CGx on venomotor tone changes in chronic AngII induced hypertension. MCFP is the pressure measured in the vasculature immediately following cardiac arrest, after pressures in all parts of the circulation are made to equilibrate, and represents the effective driving force for venous return to the heart (110). The major determinants of MCFP are compliance of the venous system and blood volume (288), and MCFP is considered the best methodology for determination of body venous tone (201).

Methods

Experimental Protocols

Radiotelemetry

Rats were acclimatized to 0.4% or 2% NaCl for 7 days and then underwent SHAM operation, CGx or RDx and a radiotelemetry transmitter was implanted. Following 10 days of recovery after surgery and a 4 day control period, an AngII or physiological saline filled osmotic minipump was implanted subcutaneously, to

deliver AngII (150ng/kg/min) or vehicle for 14 days. During the entire experimental protocol rats were allowed free access to either 0.4% NaCl or 2% NaCl diet and distilled water.

Exteriorized catheter

Rats were acclimatized to 0.4% or 2% NaCl for 7 days and then underwent SHAM operation or CGx. Six days later the rats were instrumented to allow repeated measures of MCFP. Four days of recovery were allowed after catheterization followed by a 3 day control period. AngII was then delivered subcutaneously by minipump (150ng/kg/min) for 14 days. MCFP was measured in duplicate before and starting 5 minutes after acute ganglion blockade with hexamethonium (30 mg/kg IV) on control day 2 and AngII infusion days 1, 3, 7 and 14. Each measurement of MCFP was taken 10 minutes after the previous one. Hct and PV were also measured on these days, at least 6 hours after completion of the MCFP measurements. During the entire experimental protocol rats were allowed free access to either 0.4% NaCl or 2% NaCl diet and distilled water.

Animals

A total of 16 groups of rats were initially studied to determine the effect of CGx and RDx on AngII hypertension, 8 of which were fed 2% NaCl, while the other 8 were fed 0.4% NaCl diet. For both salt diets, the groups studied to assess the effects of CGx were; CGx + AngII (n=8), SHAM + AngII (n=8), CGx + vehicle

(n=4) and SHAM + vehicle (n=4). To assess the effect of RDx the following groups were studied in rats consuming each diet; RDx + AngII (n=8), SHAM + AngII (n=8), RDx + vehicle (n=4) and SHAM + vehicle (n=4). In all groups AP was measured by radiotelemetry. Four additional groups of rats (n=7 per group) were studied to determine the effect of CGx on AngII mediated changes in venous tone. These rats were instrumented with exteriorized catheters and MCFP was measured in SHAM and CGx rats fed either 0.4% NaCl or 2% NaCl.

Results

Radiotelemetry

The effect of CGx on mean AP (MAP) response to chronic subcutaneous infusion of AngII (150 ηg/kg/min) or saline vehicle in rats implanted with radiotelemetry transmitters is shown in **figure 14**. During the control period, CGx rats had reduced MAP while eating 2% NaCl (SHAM 102 ± 1, CGx 95 ± 2 mmHg, $p<0.05$) and 0.4% NaCl diets (SHAM 99 ± 1, CGx 93 ± 1 mmHg, $p<0.05$). The hypotensive effect of CGx persisted for the duration of the experiment in the vehicle infused groups on both salt diets. CGx markedly attenuated the hypertensive response to AngII in rats on 2% NaCl diet. SHAM rats increased MAP 45 ± 6 mmHg by day 14 of AngII infusion, compared to only 19 ± 7 mmHg in CGx rats ($p<0.05$). However, CGx had little effect on the hypertensive response to AngII in rats fed 0.4% NaCl. MAP increased by 14 ± 3 mmHg in SHAM rats and 12 ± 5 mmHg in CGx rats by day 14 of AngII infusion.

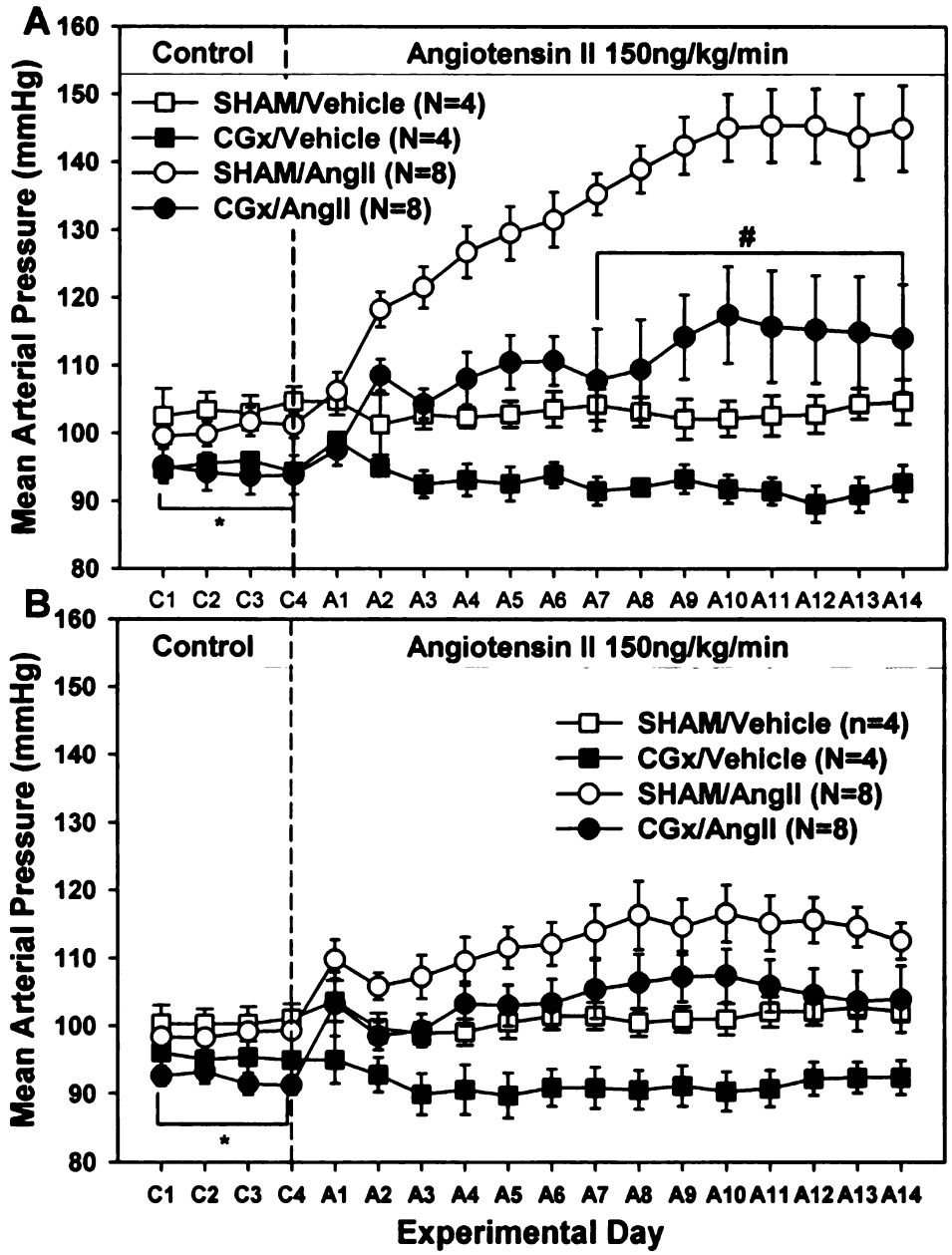


Figure 14: Effect of celiac ganglionectomy (CGx) on MAP response to infusion of AngII measured by radiotelemetry. Rats were fed either a 2% **(A)** or 0.4% **(B)** NaCl diet. * = $p < 0.05$ compared to SHAM control period. # = $p < 0.05$ compared to SHAM/AngII.

The effect of RDx on MAP response to chronic subcutaneous infusion of AngII or saline vehicle is shown in **figure 15**. Similar to the effect observed with CGx, and consistent with previous reports (126), bilateral RDx lowered MAP in normotensive rats independent of salt diet. During the control period MAP was lower in RDx rats fed 2% NaCl (SHAM 103 ± 1 mmHg, RDx 97 ± 2 mmHg, $p<0.05$) and in rats fed 0.4% NaCl (SHAM 101 ± 1 mmHg, RDx 96 ± 2 mmHg, $p<0.05$). This hypotensive effect of RDx persisted for the duration of the experiment in the vehicle infused groups on both salt diets. In contrast to the effect of CGx on the hypertensive response to AngII in rats on 2% NaCl diet, RDx had no protective effect. MAP increased in SHAM rats by 25 ± 8 mmHg compared to 31 ± 5 mmHg in RDx rats on day 14 of AngII infusion. In fact over the first 5 days of AngII infusion RDx appeared to exacerbate the pressor response to AngII. Similarly RDx had no protective effect on the AngII mediated increase in MAP in rats fed a 0.4% NaCl diet. Indeed RDx tended to exacerbate the pressor response to AngII in this group, such that MAP increased in RDx rats by 21 ± 7 mmHg compared to only 8 ± 3 mmHg in SHAM rats on day 14 of AngII infusion, however this failed to reach statistical significance ($p=0.09$).

CGx did not affect HR during the control period in rats fed 2% NaCl (CGx 423 ± 10, SHAM 423 ± 8 beats/min (BPM)) or 0.4% NaCl (CGx 411 ± 8, SHAM 418 ± 6 BPM). In SHAM rats HR decreased slightly but significantly (~25-35 BPM) in response to AngII infusion, and this was not affected by CGx (data not shown). RDx did not significantly affect HR in the control period in rats fed 2% NaCl (RDx

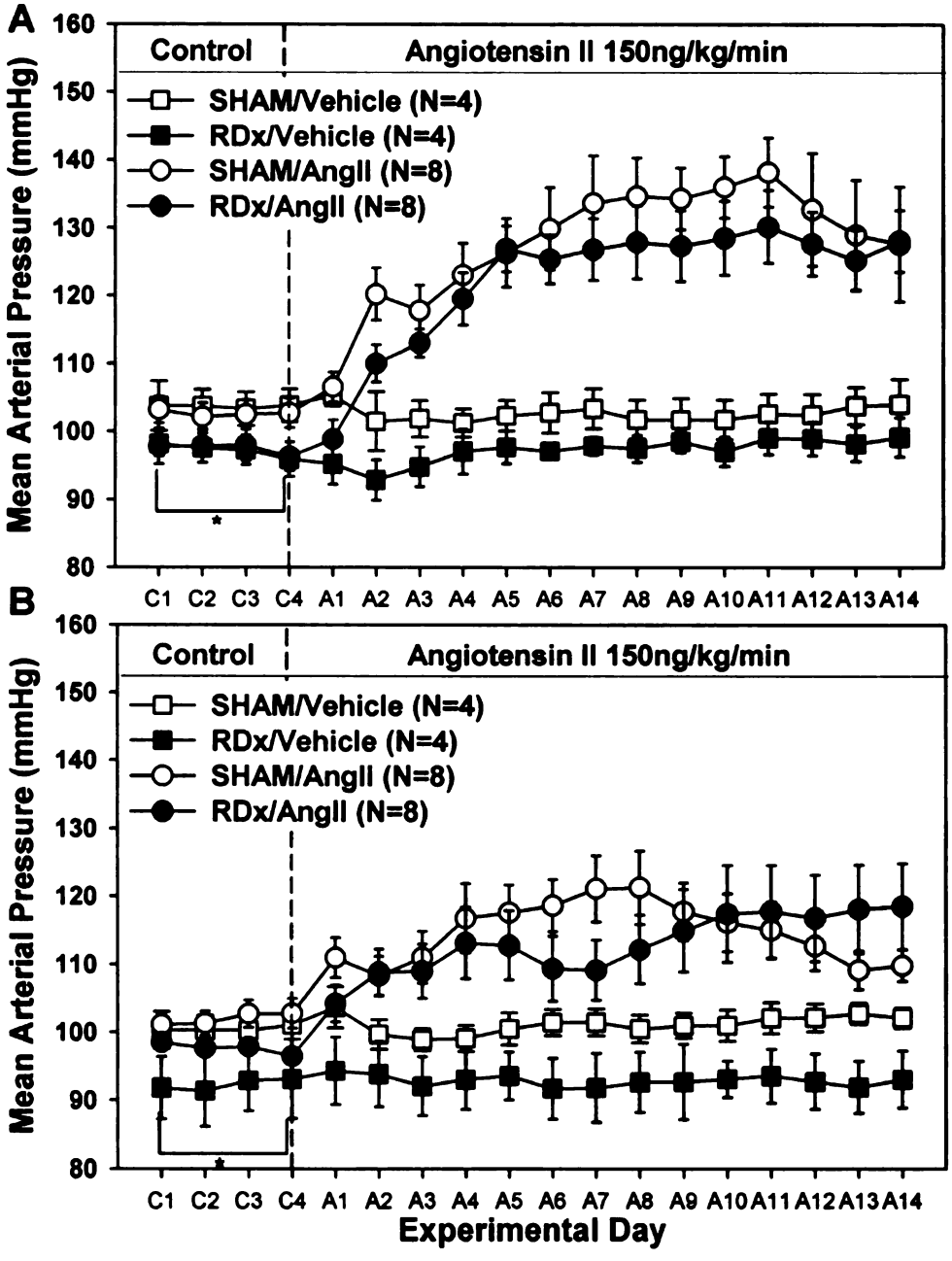


Figure 15: Effect of renal denervation (RDx) on MAP response to infusion of AngII measured by radiotelemetry. Rats were fed either a 2% **(A)** or 0.4% **(B)** NaCl diet. * = $p < 0.05$ compared to SHAM control period.

418 ± 8, SHAM 405 ± 4 BPM) or 0.4% NaCl (RDx 408 ± 6, SHAM 404 ± 6 BPM). Again HR tended to decrease during AngII infusion in SHAM rats and RDx did not significantly affect this (data not shown).

CGx did not affect body weight at the time of surgery (2% NaCl SHAM 295 ± 6, CGx 286 ± 7; 0.4% NaCl SHAM 278 ± 5, CGx 278 ± 5 g), osmotic minipump implantation (2% NaCl SHAM 344 ± 10, CGx 344 ± 7; 0.4% NaCl SHAM 325 ± 10, CGx 318 ± 9 g) or completion of the study (2% NaCl SHAM 392 ± 12, CGx 404 ± 6; 0.4% NaCl SHAM 432 ± 8, CGx 426 ± 9 g).

Exteriorized catheters

The effect of CGx on MAP response to chronic subcutaneous infusion of AngII (150 ng/kg/min) in tethered rats is shown in **figure 16**. Similar to the effect seen when AP was measured by radiotelemetry, CGx caused a significant, but mild (~7 mmHg) hypotension independent of salt intake during the control period. Consistent with the effect seen when AP was measured by radiotelemetry, CGx had little effect on chronic AngII hypertension in rats fed 0.4% NaCl, but significantly attenuated AngII hypertension in rats fed 2 % NaCl by approximately 50%. Interestingly the magnitude of the increase in AP in response to AngII infusion was significantly greater in tethered animals compared to radiotelemetry animals by approximately 25 mmHg, even though control period AP was indistinguishable between the AP measurement methods. CGx did not affect HR during the control period in rats fed 2% NaCl (CGx 387 ± 13, SHAM 370 ± 8

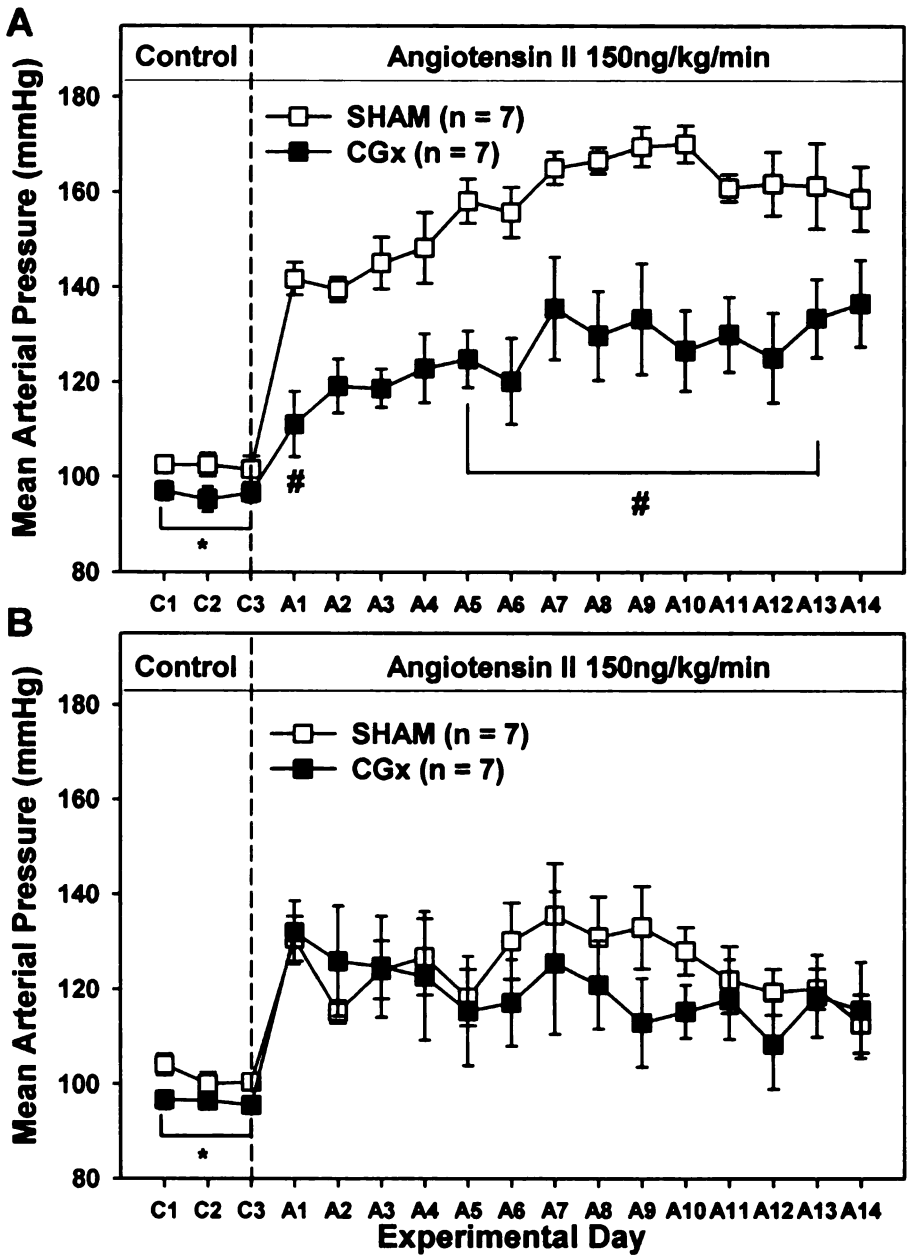


Figure 16: Effect of CGx on MAP response to AngII infusion in catheterized animals. Rats were fed either a 2% **(A)** or 0.4% **(B)** NaCl diet. * = $p < 0.05$ compared to SHAM control period. # = $p < 0.05$ compared to SHAM/AngII

BPM) or 0.4% NaCl (CGx 383 ± 10, SHAM 376 ± 8 BPM), and did not affect the transient decrease in HR in response to AngII infusion (data not shown).

Figure 17 shows MCFP and BV responses to chronic subcutaneous infusion of AngII in SHAM and CGx operated rats measured on control day 2 and AngII infusion days 1, 3, 7 and 14. Surprisingly, in the control period, BV and MCFP were unaffected by CGx in rats fed either 0.4% or 2% NaCl. There were no statistically significant changes in BV from the control period in response to AngII infusion in any group. Consistent with our previous study (140) MCFP was unchanged in response to AngII infusion in SHAM animals fed 0.4 % NaCl, however there was a significant and marked increase in MCFP (~ 3 mmHg) for the duration of AngII infusion in SHAM rats fed a 2% NaCl diet. CGx had no effect on MCFP responses to AngII in rats fed 0.4% NaCl, but completely prevented MCFP increases in animals fed 2% NaCl.

The peak fall in MAP in response to acute ganglion blockade with hexamethonium (30 mg/kg IV) is shown in **figure 18** along with the fall in MCFP 5 minutes after hexamethonium administration. CGx did not alter peak depressor responses to ganglion blockade in animals fed 2 % NaCl during the control period. However, quite surprisingly, CGx significantly enhanced the peak depressor response to hexamethonium in rats fed 0.4% NaCl during this control period compared to SHAM animals. Consistent with our previous work (140)

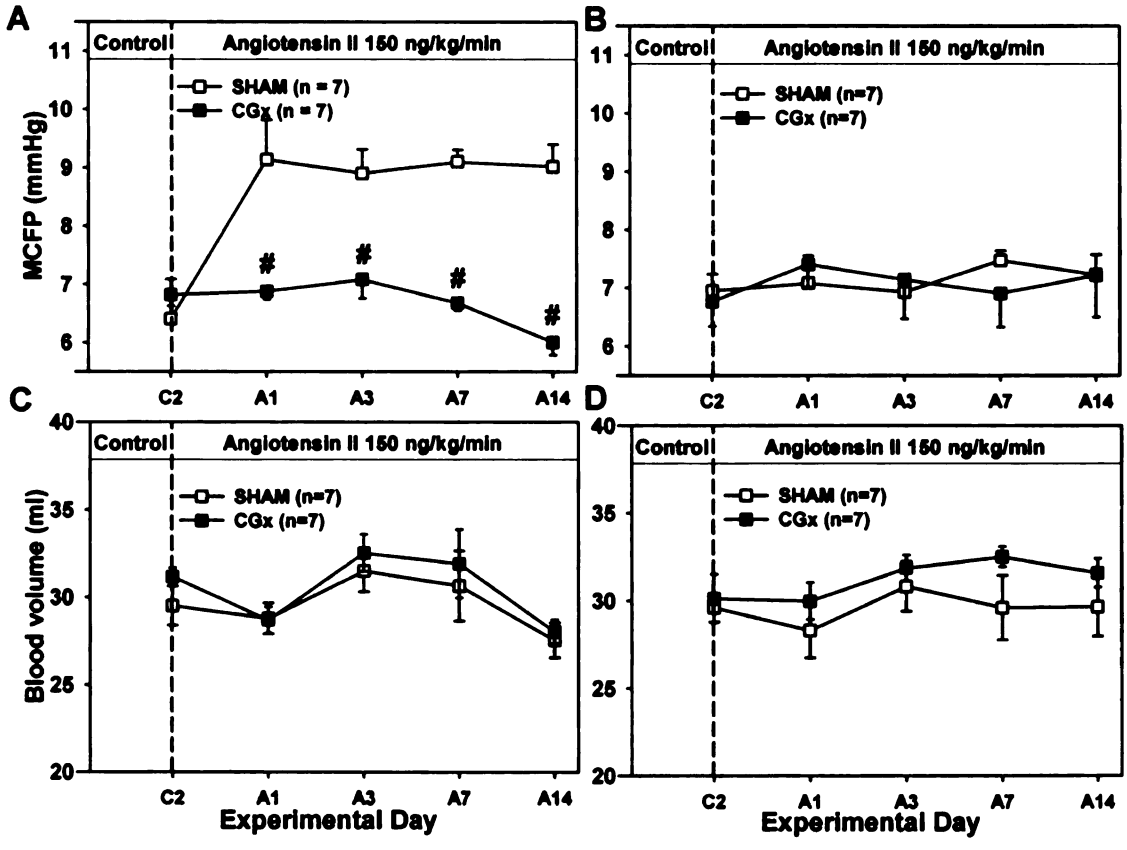


Figure 17: Effect of CGx on blood volume and MCFP response to AngII infusion in catheterized animals. MCFP response to infusion of AngII in SHAM operated or CGx rats fed either a 2% **(A)** or 0.4% **(B)** NaCl diet and blood volume response to AngII infusion in SHAM operated or CGx rats fed either a 2% **(C)** or 0.4% **(D)** NaCl diet. # = $p < 0.05$ compared to SHAM.

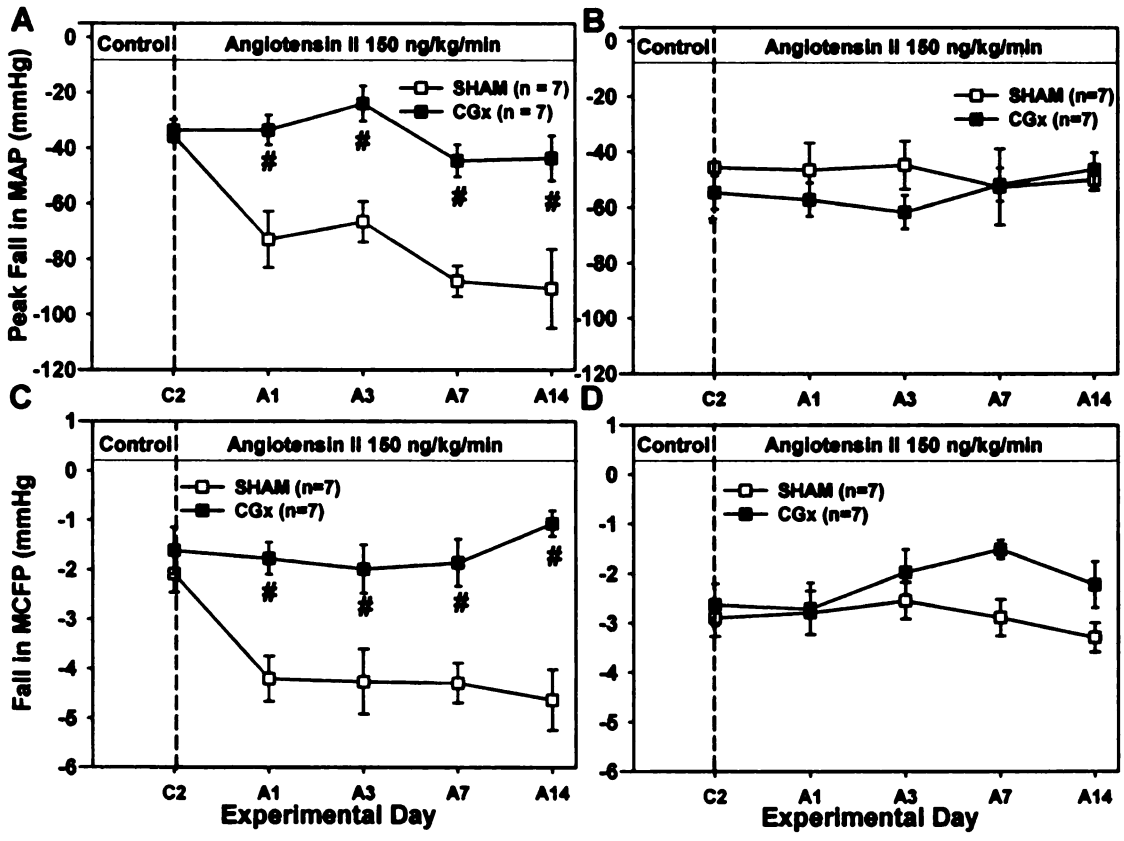


Figure 18: Hemodynamic response to acute ganglion blockade in catheterized CGx animals during AngII infusion. Peak fall in MAP in SHAM operated or CGx rats fed either a 2% **(A)** or 0.4% **(B)** NaCl, and decline MCFP measured 5 minutes after hexamethonium in rats fed either a 2% **(C)** or 0.4% **(D)** NaCl. * = $p < 0.05$ compared to SHAM control period. # = $p < 0.05$ compared to SHAM/AngII

depressor responses to ganglion blockade were not changed during AngII infusion in SHAM rats fed 0.4% NaCl; CGx did not effect this. However there were significant and marked increases in MAP depressor response to hexamethonium during AngII infusion, in rats fed a 2% NaCl diet. This increase was significant on day 1 of AngII infusion, remained statistically significantly increased on day 3 and was further increased on days 7 and 14 of AngII infusion. Remarkably, CGx clearly attenuated (but did not completely abolish) these increased falls in MAP in response to hexamethonium administration. Again consistent with our previous published work (140) the fall in MCFP 5 minutes after ganglion blockade was enhanced during AngII infusion in SHAM animals fed 2% NaCl, but not in SHAM animals fed 0.4% NaCl. This increased sympathetic component contributing to the elevated MCFP in SHAM rats fed 2% NaCl was completely prevented by CGx.

Verification of denervation

Tissue NE content of the abdominal organs, verifying successful CGx and bilateral RDx, is presented in **figure 19**. First it should be noted that AngII infusion alone affected tissue NE content. In SHAM operated rats fed 2% NaCl diet AngII infusion alone significantly ($p<0.05$) decreased NE content of the left and right kidney and spleen compared to vehicle, by approximately 36%, 42% and 30% respectively. Small intestine and liver NE was unaffected by AngII infusion in SHAM rats fed 2% NaCl. In SHAM rats fed 0.4% NaCl diet AngII infusion also tended to decreased NE content of both kidneys and spleen.

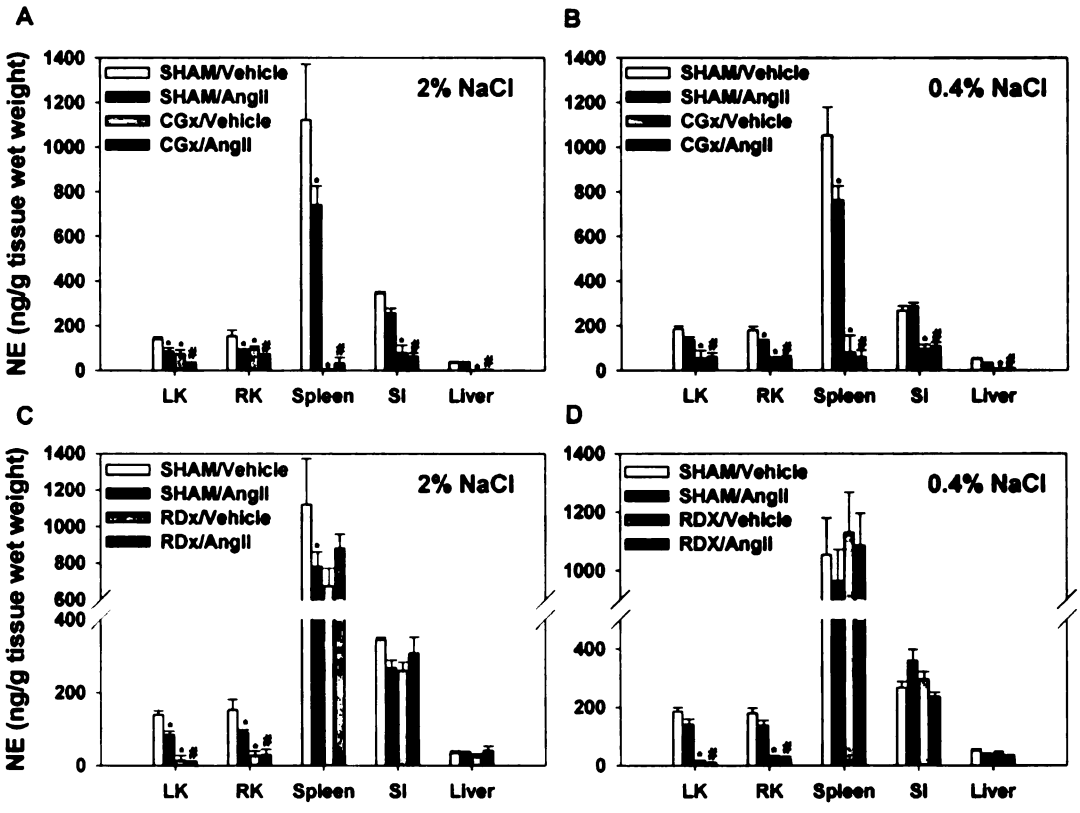


Figure 19: Verification of denervation by CGx and RDx. Tissue NE content (ηg NE/ g tissue) in the splanchnic organs in response to CGx in rats fed 2% NaCl **(A)** or 0.4% NaCl diet **(B)** and RDx in rats fed 2% NaCl **(C)** or 0.4% NaCl diet **(D)**. * = $p < 0.05$ compared to SHAM vehicle infused rats. # = $p < 0.05$ compared to SHAM AngII infused rats.

However, this decrease was only statistically significant ($p<0.05$) in the right kidney and spleen of the SHAM control group for CGx. Small intestine and liver NE was unaffected by AngII infusion in SHAM rats fed 0.4% NaCl. Surgical CGx and RDx caused consistent and predictable decrements in tissue NE content. In rats fed a 2% NaCl diet, CGx resulted in a significant ($p<0.05$) reduction in NE content of the left and right kidneys, spleen, small intestine and liver, by approximately 50%, 31 %, 98%, 76% and 95% respectively. In rats fed a 0.4% NaCl diet, CGx resulted in a significant ($p<0.05$) reduction in NE content of the left and right kidneys, spleen, small intestine and liver, by approximately 61%, 60 %, 92%, 64% and 86% respectively. In rats fed a 2% NaCl diet, RDx resulted in a significant ($p<0.05$) reduction in NE content of the left and right kidneys, by approximately 90%, and 85 % respectively, but did not affect NE content of the spleen, small intestine or liver. Similarly in rats fed a 0.4% NaCl diet, RDx resulted in a significant ($p<0.05$) reduction in NE content of the left and right kidneys, by approximately 94%, and 85 % respectively, but did not affect NE content of the spleen, small intestine or liver.

Discussion

The major new finding of this study is that selective removal of sympathetic innervation to the splanchnic circulation markedly attenuated AngII-salt hypertension in the rat. This novel result was verified using two different techniques, radiotelemetry and exteriorized catheters, to measure AP directly. The observation that CGx attenuated the hypertensive response to AngII only in

the presence of a high salt diet supports prior conclusions that the increment in hypertensive response to AngII seen in rats on a high salt diet is sympathetically driven (18, 19, 140, 194, 231, 259). In contrast, bilateral RDx had no protective effect on AngII hypertension. In fact, RDx tended to exacerbate the hypertension, especially in rats on normal salt diet. This is consistent with previously reported baroreflex mediated decreases in renal nerve activity in AngII hypertension (10, 11, 164, 165), since a sustained decrease in renal nerve activity should reduce the hypertensive response to AngII infusion (165). Therefore the lack of a protective effect of RDx in this model is not surprising.

CGx not only significantly attenuated AngII-salt mediated increases in AP, but also moderated enhanced AP responses to hexamethonium and completely abolished the neurogenically mediated increase in MCFP seen in AngII-salt hypertension. In the absence of a change in blood volume, increases in MCFP represent an increase in venomotor tone (83, 201, 288). In the present study blood volume was unchanged. Together, this suggests that in the presence of a high salt diet AngII activates the splanchnic SNS to increase venomotor tone and AP.

To fully characterize the effect of CGx, the splanchnic organs were harvested immediately following completion of the experimental period and tissue NE content was measured as an index of sympathetic denervation. CGx almost completely abolished NE in the spleen and liver and caused a marked reduction

in small intestinal NE. These findings verify successful sympathetic denervation to the splanchnic bed. CGx also reduced renal NE content by 30-60%. The renal nerves have previously been implicated in the pathogenesis of various forms of experimental hypertension, including AngII hypertension (126, 132, 160, 187, 192, 286). Therefore selective bilateral RDx was a critical control group to determine if CGx could be exerting its effects by RDx. Since RDx did not attenuate the hypertension in response to AngII infusion it is clear that CGx was not exerting its antihypertensive effects via disruption of renal sympathetic innervation. Anatomically in the rat, the renal nerves are in close proximity to the celiac plexus and the liberal use of phenol to destroy the renal nerves could inadvertently damage the celiac plexus, disrupting splanchnic sympathetic innervation. Other studies using RDx to investigate the roles of the renal nerves in rat models of hypertension did not include comprehensive evaluation of the effect of RDx on non-renal splanchnic innervation. Therefore it is not clear whether some of the effects previously attributed to RDx could have been the result of CGx. This is the first study to exclude an effect of bilateral RDx on tissue NE content in non-renal splanchnic organs.

The differential effects of CGx and RDx also suggest that SNS activation in response to AngII, in rats fed a high salt diet, is regionally heterogeneous. Regionalized sympathetic activation has been convincingly demonstrated by direct nerve recordings in rabbits (215) and Esler's group has elucidated the importance of regionalized sympathetic activation in human cardiovascular

disease by using NE spillover techniques (2, 62, 67, 68, 70). The finding in this study that CGx markedly attenuated enhanced depressor responses to ganglion blockade during AngII-salt hypertension implies that the majority of the sympathetic activation in this model is directed towards the splanchnic bed and supports the possibility of regionalized sympathetic activation. Although our results are consistent with differential sympathetic activation, this hypothesis needs to be tested directly using the complementary methods of direct sympathetic nerve recordings and regional NE spillover measurements.

The demonstration of the importance of splanchnic SNS activity to the pathogenesis of hypertension in this study may explain the historical success of surgical thoracolumbar splanchnicectomy in prolonging survival times in patients with essential hypertension (242), in particular those refractory to medical management (275). Interestingly a recent study showed that poorly controlled essential hypertension in human patients was markedly improved in patients after bilateral T_3 endoscopic sympathetic block (41). Although the mechanism of the effect is unclear, it is possible that the beneficial effects are mediated through inhibition of splanchnic sympathetic nerve activity.

Another new finding in this study is that, similar to bilateral RDx, CGx chronically lowers AP independent of salt intake in normotensive rats during the control period. Surprisingly, this hypotension was not associated with a concurrent reduction in MCFP. Therefore it appears that the hypotensive effect of CGx

during the control period is not mediated through changes in whole body vascular capacitance. The fall in basal MAP could be a result of changes in the resistance bed of the splanchnic circulation. Additionally, although the fall in MCFP in response to ganglion blockade was slightly less in CGx animals during the control period, it was not significantly different from SHAM animals. This indicates that there is non-splanchnic neural compensation to maintain basal MCFP. However it appears as though the compensating neural bed responsible for maintaining a normal MCFP after CGx is not activated in AngII-salt hypertension as MCFP does not rise in these animals. The AP lowering effects of CGX have also been reported in sheep (113). Transient, but severe hypotension and orthostatic hypotension are also documented side effects of celiac plexus neurolysis for the treatment of pancreatic malignancies in humans (58, 84, 173, 191, 290). This emphasizes the importance of SNS innervation to the splanchnic vascular bed in the control of blood pressure under normal conditions.

Other than mild hypotension, CGx was well tolerated in this study and no other adverse effects were observed. The metabolic effect of selective removal of splanchnic innervation in rats has previously been comprehensively evaluated (92). Bilateral splanchnic nerve section caused no obvious behavioral signs of distress and no differences in body weight, daily food intake, abdominal fat, brown adipose tissue weight, abdominal organ weight, plasma leptin or hypothalamic neuropeptide Y content compared to sham operated control rats (92). Experimental evidence also indicates the chronic extrinsic denervation of

the small intestine does not affect net intestinal absorption of water and electrolytes (57, 87, 92, 112, 116, 161, 162, 229, 230, 256, 263, 269, 293). We have also shown that CGx does not affect Na+ intake in rats fed 2% NaCl (198).

The exact hemodynamic mechanism by which CGx is affects AngII-salt hypertension is still not completely clear and needs to be investigated further by chronic instrumentation of rats for measurements of cardiac output and total peripheral resistance determination. Neurogenically mediated increases in venous tone during AngII-salt hypertension, which are completely prevented by CGx, may contribute to the pathogenesis of hypertension in this experimental model by causing a venous to arterial translocation of blood volume, without a change in total vascular volume (140). The arterial circulation in the rat is approximately 60 times less compliant than the venous system (288). A venous to arterial translocation of only a small volume of blood, in the presence of an impairment in renal excretory function, could significantly increase AP (106, 108, 172). This increase in AP may initiate a series of changes in the arterial system, including increased myogenic vascular tone and expression of voltage gated calcium channels (209, 243), that facilitate the maintenance and progression of elevated AP by increasing TPR. CGx, which prevents the observed neurogenically mediated venoconstriction in the AngII-salt model, may prevent the venous to arterial translocation of blood and subsequent increase in TPR. However, in addition to removal of splanchnic venous sympathetic innervation, CGx also removes sympathetic arterial innervation and it is therefore unclear

which has the greatest hemodynamic importance. Retrograde labeling experiments have shown that the majority of neurons in the celiac ganglion supplying the vasculature dually innervate veins and arteries (120). In fact only 5% of neurons solely innervate the veins (120), making it impossible at this time to test our hypothesis using selective venous denervation.

Another finding of this study worth noting is that the hypertensive response to chronic AngII infusion is dependent on the AP measurement method; a finding consistent with our unpublished observations and those of others (206). AP was indistinguishable during the control period irrespective of measurement technique, but the magnitude of the increase in AP in response to AngII infusion was significantly greater in tethered animals. Exteriorized catheters and tethering have been proposed to introduce a stress on the animal (152), and from our study it appears as though the stress of tethering, while alone is not sufficient to influence AP, is acting to sensitize the animal to external stimuli such as AngII.

Perspectives

Compelling evidence indicates that SNS activation may be a common mechanism of hypertension in human essential hypertension and many experimental models. The identification of the splanchnic vascular bed as a critical target in AngII-salt hypertension greatly improves our understanding of peripheral neural mechanisms leading to hypertension. Unveiling the genomic and electrophysiologic basis of AngII-salt induced dysregulation of

neurotransmission through the celiac plexus, which allows for increased sympathetic activity to the splanchnic vasculature, may identify novel therapeutic targets for the treatment of hypertension. Centrally acting sympatholytics have been demonstrated to be effective in the treatment of essential hypertension, but are poorly tolerated due to their side effect profile. A peripheral neural target, such as the celiac plexus, may therefore represent a more desirable target for therapeutic intervention.

CHAPTER FIVE: REGIONAL SPLANCHNIC HEMODYNAMICS IN CHRONIC ANGIOTENSIN II – SALT HYPERTENSION IN THE RAT

The splanchnic sympathetic nervous system (SNS) appears to be a critical neural target in chronic angiotensin II (AngII) – salt hypertension. Celiac ganglionectomy (CGx), to selectively disrupt sympathetic innervation to the splanchnic organs, prevents AngII-salt mediated increases in venous smooth muscle tone (139), significantly attenuates AngII-salt hypertension (141) and essentially abolishes the chronic vasoconstrictor responses to AngII in rats consuming a high salt diet (197). To further investigate the role of the splanchnic circulation on the hemodynamic profile of chronic AngII-salt hypertension, regional splanchnic hemodynamics were studied.

Arterial pressure (AP) can be increased by one of two general mechanisms; either an increase in blood flow or an increase in vascular resistance (45). The regional splanchnic hemodynamic pattern, which accompanies AngII mediated increases in AP, that would be most consistent with sympathetic activation to the splanchnic bed is an increase in splanchnic resistance without an increase in splanchnic blood flow. To test this hypothesis, non-hepatic splanchnic blood flow was approximated by directly measuring portal blood flow chronically in conscious rats using a transit-time ultrasound perivascular flow probe. This technique has previously been extensively validated (47).

Methods

Experimental protocol

Rats consuming a high salt diet were instrumented with an arterial catheter and perivascular flow probe on the portal vein. Following 10 days of recovery and a four day control period, AngII (150 ng/kg/min) was delivered subcutaneously by osmotic minipump. The minipump was removed and the rats studied again after four days of recovery.

Animals

A total of 5 rats were used in this study, all of which consumed a 2% NaCl diet and were infused with AngII.

Results

Regional splanchnic hemodynamics in chronic AngII-salt hypertension are shown in **figure 20**. MAP increased significantly for the entire duration of AngII infusion. MAP was 101± 2 mmHg in the control period and increased to 158 ± 9 mmHg on day 14 of AngII infusion. MAP return to control levels (102 ± 6) mmHg within four days of ending AngII infusion. Surprisingly, heart rate was statistically unaffected by AngII infusion. Portal blood flow was stable during the control period and averaged 18 ± 2 ml/min. Portal flow tended to decrease for the entire duration of AngII infusion and then return to control levels within four days of ending AngII (19 ± 1 ml/min). However, portal flow was only significantly reduced on AngII infusion day 1 (12 ± 4 ml/min). Splanchnic resistance was increased for the entire duration of AngII infusion, but this increase was only statistically significant on

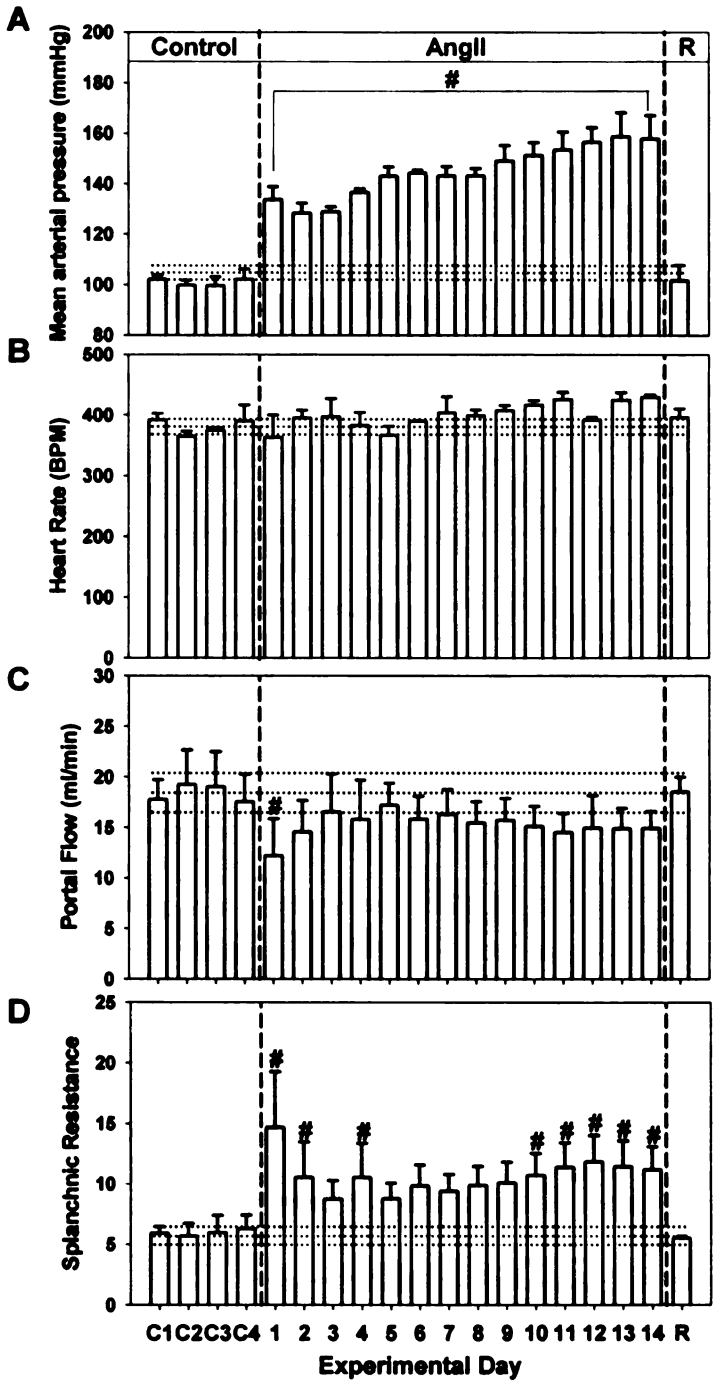


Figure 20: Regional splanchnic hemodynamics in chronic AngII-salt HTN. MAP (A), HR (B), portal blood flow (C) and splanchnic resistance (D) during control, AngII and recovery (R) periods in rats fed a high salt diet. # = $p < 0.05$ compared to control period.

AngII infusion days 1, 2, 4, and 10-14. Splanchnic resistance returned to baseline levels within 4 days of stopping AngII.

Discussion

The findings of this study are consistent with the hypothesis that splanchnic SNS activity is increased in chronic AngII-salt hypertension. Direct measurements of portal blood flow in combination with the measurement of AP, documented that increased splanchnic vascular resistance was accompanied by a reduction in splanchnic blood flow in response to chronic infusion of AngII in rats consuming a high salt diet. This hemodynamic profile suggests active vasoconstriction in the splanchnic circulation. This splanchnic vasoconstriction is likely neural in origin, given that selective splanchnic denervation prevents AngII-salt mediated increases in venous smooth muscle tone (139), significantly attenuates AngII-salt hypertension (141) and essentially abolishes the chronic systemic vasoconstrictor responses to AngII in rats consuming a high salt diet (197). However, a direct vasoconstrictor effect of AngII or an autoregulatory response cannot be entirely discounted at this time, although, splanchnic myogenic responses, particularly that of splenic vessels, are relatively weak (16). Measurements of splanchnic NE spillover and assessing the effect of CGx on regional splanchnic hemodynamics would further test the hypothesis that splanchnic vasoconstriction in chronic AngII-salt hypertension is sympathetic in origin.

In this study, splanchnic vascular resistance was observed to increase on the first day of AngII infusion, implying a hemodynamically important role in the pathogenesis of the hypertension that develops. Interestingly, a recent study demonstrated that vascular resistance increases in the hepatosplanchnic circulation before any other bed in humans with borderline hypertension (248) indicating the splanchnic SNS may also be important in the pathogenesis of human hypertension.

The calculation of splanchnic resistance made in this study, is based on the premise that portal blood flow provides an accurate assessment of splanchnic blood flow. A major reason that the hemodynamic contribution of the splanchnic bed to hypertension is relatively unclear is due to difficulties of accessibility (59). Unlike the anatomical blood supply to non-splanchnic beds, there is no one vascular segment that provides all arterial delivery to the splanchnic bed. For example, assessment of regional renal hemodynamics is relatively straightforward, as measurements of renal artery blood flow detect all arterial delivery to the kidney. However, the anatomical vascular supply of the splanchnic circulation is considerably more complex. The splanchnic circulation is composed of gastric, small intestinal, colonic, pancreatic, hepatic and splenic circulations arranged in parallel (202). The major arterial supply to the splanchnic bed is via the celiac, superior mesenteric and inferior mesenteric arteries, all of which arise separately from the aorta. Therefore measuring total arterial flow to the splanchnic circulation is problematic. The portal vein provides venous drainage

from the from the gastric, small intestinal, pancreatic, splenic and the majority of the colonic circulations (202), and therefore probably provides the most complete assessment of non-hepatic splanchnic flow. It should be emphasized that hepatic flow is not included in measurements of portal venous flow.

Perspectives

The splanchnic circulation is densely innervated by the SNS and the level of sympathetic tone represents the most important regulator of splanchnic vascular resistance. Non-hepatic splanchnic vascular resistance significantly increases early on in the development of experimental and human hypertension. This would be best explained by an increase in splanchnic SNS activity.

CHAPTER SIX

King AJ, Novotny, M, Swain, G and Fink GD. Whole body norepinephrine kinetics in angiotensin II – salt hypertension in the rat. *Am J Physiol – Regul Integr Comp Physiol*, February 6, 2008. (used with permission)

Sympathetic nervous system (SNS) activity is commonly increased in human essential hypertension (104). The most compelling evidence for SNS activation in hypertensive humans comes from direct nerve recordings of muscle sympathetic nerve activity (MSNA) (3, 98, 233) and measurements of whole body and regional norepinephrine (NE) spillover (65, 67, 70, 72, 81). While SNS activation is also thought to be a common feature of many experimental animal models of hypertension, direct measures of SNS activity are relatively lacking. Indirect evidence for SNS activation includes enhanced depressor responses to ganglion or adrenergic receptor blockade, and regional denervation and central nervous system lesion studies (6, 20, 24, 82, 97, 139, 141, 190, 251). A number of elegant studies have utilized direct recordings of sympathetic nerve activity to demonstrate increased single fiber activity to the kidney in spontaneously hypertensive rats (SHR) and increased splanchnic nerve activity in rats with chronic AngII hypertension (167, 257). However these measurements were often made for short durations under anesthesia or after short recovery periods from surgery. Measurements of NE spillover allow for repeated assessment of sympathetic activity in conscious, undisturbed animals. The purpose of this study was to investigate total body NE kinetics, in a rat model of chronic angiotensin II (AngII) - salt hypertension, as an index of global sympathetic outflow.

Since Esler and colleagues first applied radioisotope dilution principles for measurements of NE release and clearance in humans (66), it has become clear that these methods provide a more accurate assessment of sympathetic

transmitter release than allowed by measurements of plasma catecholamines alone (59, 71, 265). The major advantage of whole body NE spillover as an index of global sympathetic outflow is that the dynamic processes of NE clearance and NE spillover can be distinguished when analyzing plasma NE levels (63). Although these techniques have been extensively applied to characterize sympathetic activity in numerous human cardiovascular and metabolic diseases, they have been used sparingly in experimental animal models of hypertension. However methods to apply these techniques to laboratory animals have been described (136).

Angiotensin type 1 (AT1) receptor antagonists have been shown to significantly reduce MSNA in lean and obese hypertensive humans (12, 99), implicating AngII in the pathogenesis of SNS activation in human hypertension. We have used depressor responses to acute ganglion blockade with hexamethonium (139, 141) and regional denervation techniques (141) as indirect evidence for sympathetic activation in a rat model of chronic AngII hypertension. That evidence suggested, however, that sympathoexcitation to AngII only occurs in rats fed a high salt diet. Therefore in the current experiments we specifically tested the hypothesis that global SNS activity is increased in chronic AngII-salt hypertension only in rats ingesting a high salt diet.

Methods

Experimental Protocol

Rats were acclimatized to 0.4% NaCl (n=8) or 2% NaCl (n=9) diet for 7 days and then exteriorized arterial and venous catheters were implanted. Five days of recovery were allowed after catheterization followed by a 3 day control period. AngII (150ng/kg/min) was then delivered subcutaneously by osmotic minipump for 14 days. Endogenous plasma NE levels and total body NE spillover and clearance were determined on control day 3 and AngII infusion days 7 and 14. Rats were allowed free access to either 0.4% NaCl or 2% NaCl diet and distilled water for the duration of the experiment.

Animals

A total of 17 rats were used in this study. Nine were fed 2% NaCl and 8 were on 0.4% NaCl. All were infused with AngII.

Results

The MAP and HR response to chronic infusion of AngII in animals fed 0.4% NaCl or 2% NaCl is shown in **figure 21**. Both MAP (0.4% NaCl 106 ± 3, 2% NaCl 103 ± 2 mmHg) and HR (0.4% NaCl 395 ± 5, 2% NaCl 372 ± 4 beats/min) were unaffected by a high salt diet during the control period. AngII caused a significant ($p<0.01$) increase in MAP for the entire duration of infusion, in rats fed both 0.4% NaCl and 2% NaCl. However, the effect of AngII to increase MAP was dependent on salt intake, as indicated by a statistically significant ($p<0.01$) interaction (two-way mixed design ANOVA) between the two factors. MAP increased to a greater

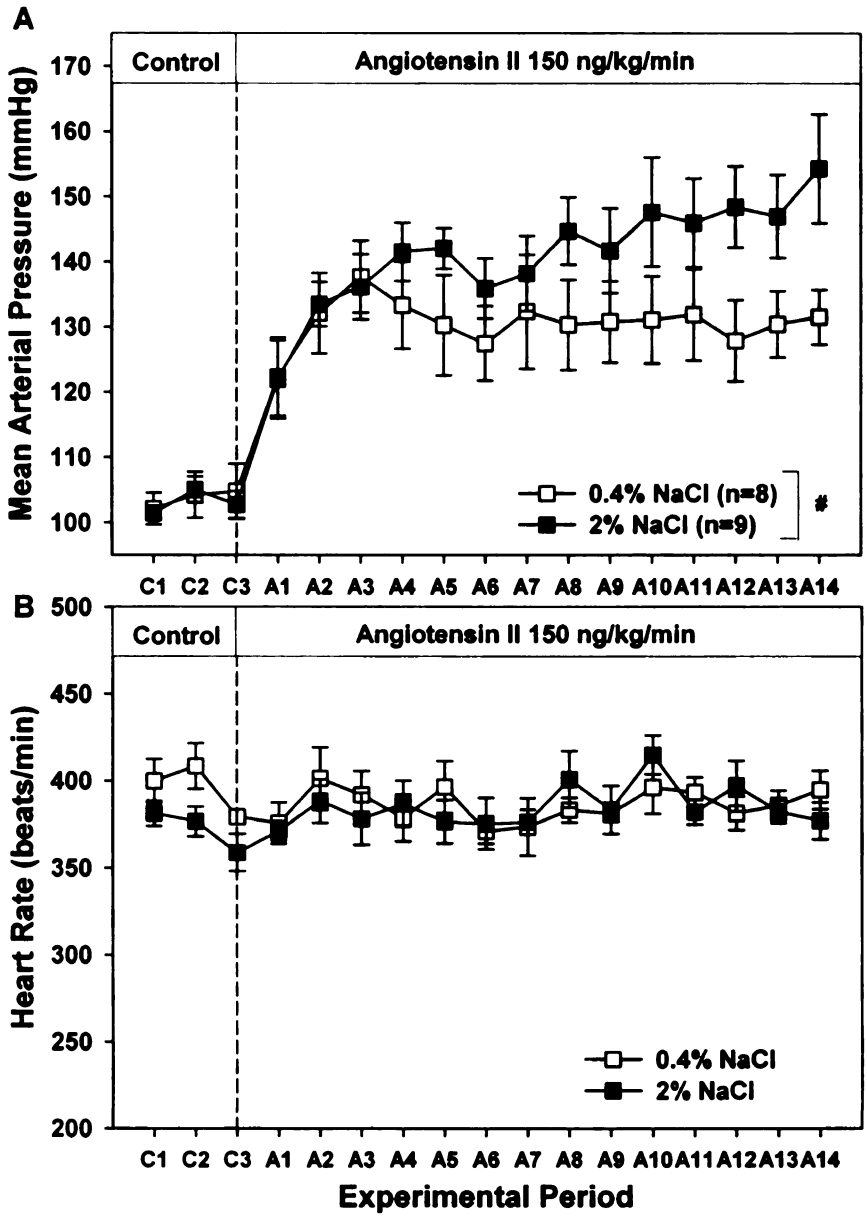


Figure 21: MAP and HR responses to AngII infusion in rats catheterized rats instrumented for NE spillover. MAP **(A)** and HR **(B)** responses in rats fed 0.4% or 2% NaCl. # = interaction $p < 0.05$ two-way ANOVA.

level in animals fed 2% NaCl. HR responses to AngII infusion were similar in rats fed both salt diets.

Infusion of 0.13 μCi/min/kg of ^{3}H-NE for 90 minutes resulted in an average steady-state plasma concentration of ^{3}H-NE of 1908 dpm/ml. The specific activity of the infused ^{3}H-NE was 525 dpm/pg. Therefore our infusion protocol increased total plasma NE concentration by an average of 3.6 pg/ml. The average plasma NE concentration measured in the study was 215.25 pg/ml. Therefore ^{3}H-NE contributed on average only 1.6% of the total NE measured. We also determined that the infusion of ^{3}H-NE at this rate did not affect MAP or HR.

Figure 22 shows plasma NE and total body NE clearance and spillover during the control period and on AngII infusion days 7 (A7) and (14) in rats fed a 0.4% or 2% NaCl diet. During the control period, plasma NE tended to be lower in rats on high salt ($p=0.09$), whereas NE clearance tended to be higher in high salt rats ($p=0.06$), however these differences failed to meet the criterion for statistical significance. As a result NE spillover was similar, independent of salt diet, during the control period. In rats fed 0.4% NaCl plasma NE, NE spillover and NE clearance were unchanged by AngII infusion. In contrast however, in rats on 2% both plasma NE and NaCl, plasma NE and NE spillover increased significantly ($p<0.05$) during AngII infusion, whereas NE clearance was unchanged. Two-way ANOVA identified a statistically significant ($p<0.05$) interaction between AngII mediated changes in NE spillover, and salt intake, whereas salt intake did not

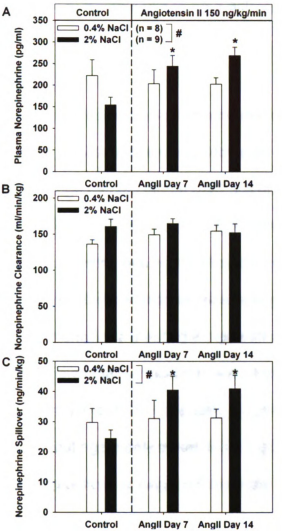


Figure 22: Plasma NE **(A)**, NE clearance **(B)** and NE spillover **(C)** in response to AngII infusion. Rats fed 0.4% or 2% NaCl. * = $p < 0.05$ versus control period. # = interaction $p < 0.05$ two-way ANOVA.

affect the response of clearance to AngII. This demonstrates that a high salt diet modulates the ability of AngII to activate global SNS activity.

Discussion

A high salt diet alone or AngII infusion in rats fed a normal salt diet did not increase global sympathetic outflow in this study. However when administered in combination, high salt and AngII caused global SNS activation associated with a further increase in arterial pressure. The finding of sympathoactivation in chronic AngII hypertension only in the setting of a high salt diet is consistent with our previous work showing enhanced depressor responses to ganglion blockade during chronic AngII infusion only in rats eating a high salt diet (139, 141). In addition we have recently shown that surgical removal of the celiac plexus to selectively disrupt splanchnic sympathetic innervation significantly attenuated chronic AngII hypertension, but again only in rats fed a high salt diet (141). Therefore the SNS activating actions of AngII in this model appear critically dependent on the presence of a high salt diet.

In this study we documented global SNS activation in rats fed 2% NaCl but not rats fed 0.4% NaCl on day 7 of AngII infusion despite similar increases in MAP at this time point. Therefore it appears while the magnitude of AngII induced hypertension is similar by day 7 the underlying mechanism must be different depending on salt intake. Under conditions of a normal salt diet AngII seems to be acting to increase MAP by non-neural mechanisms. While the exact nature of

these mechanisms is unclear, it may involve the direct vasoconstrictor or renal actions of AngII. In contrast, in rats fed a high salt diet neurogenic mechanisms of hypertension appear to predominate. Studies have demonstrated AngII can increase sympathetic outflow by a central action, but also has stimulatory effects on sympathetic ganglia, including the adrenal medulla and can facilitate neurotransmission at the neuro-effector junction via both pre-synaptic and post-synaptic actions (217). The results of this study are consistent with the actions of AngII at any level of the SNS.

Reports on the ability of AngII to activate the SNS in humans are conflicting. Studies show that AT1 receptor antagonists significantly reduce MSNA in lean (12) and obese (99) hypertensive humans and that AT1 receptor blockade or angiotensin converting enzyme inhibition reduces MSNA in hypertensive patients with chronic kidney disease (185). However Esler's group combined microneurography and radioisotope dilution methodology in a randomized, placebo-controlled crossover study to demonstrate that MSNA and whole body NE spillover were unchanged by AT1 receptor antagonism in human essential hypertension, and concluded that the blood pressure lowering actions of AT1 blockade are not related to sympathoinhibition (150). The evidence for AngII mediated sympathoactivation in experimental animals is also controversial. For example Luft and colleagues attenuated chronic AngII hypertension in the rat by adrenergic blockade and showed significant increases in splanchnic nerve activity in conscious AngII infused rats instrumented with splanchnic nerve

electrodes (167). However Kline showed no significant differences in NE turnover in the heart, kidney, skeletal muscle or intestine in AngII hypertensive rats (143) indicating that SNS activity was not increased, although depressor responses to ganglion blockade were significantly larger in the AngII infused rats (143). Therefore it appears as though the effect of AngII on SNS activity depends on the specific human population or the experimental conditions under which it is studied. It is the hypothesis of our group that one of the conditions promoting AngII mediated sympathoactivation is a high salt diet, and potential mechanisms mediating this interaction have been reviewed recently (196).

Other studies, using measurements of plasma catecholamines as an index of sympathetic activity, have also suggested that salt enhances AngII mediated SNS activation in the rat (231). Recently Malpas and colleagues showed in rabbits that the effects of AngII on SNS activity were dependent on the dose of AngII as well as dietary salt intake (181). The ability of salt to enhance SNS activation in experimental animal models of hypertension is not unique to AngII. Brooks and coworkers have elegantly demonstrated using direct recordings of lumbar sympathetic nerve activity that the elevated blood pressure and sympathoactivation seen in deoxycorticosterone acetate (DOCA) – salt hypertension in the rat is driven by increased NaCl levels (189). Elevated osmolality appears to be acting centrally in the brain to support increased blood pressure and SNS activation in the DOCA-salt model, as bilateral intracarotid infusion, but not intravenous administration, of hypotonic fluid rapidly decreased

blood pressure in DOCA-salt rats, and this fall in blood pressure was partially prevented by ganglion blockade (190). A high salt diet also stimulates SNS activity in spontaneously hypertensive rats (38, 145) and Dahl salt sensitive rats (94, 147).

More importantly there is considerable evidence that neurogenic mechanisms may play a role in the pathophysiology of salt sensitivity in human essential hypertension. The phenomenon of salt-sensitivity, an increase in blood pressure with increasing sodium intake, is common in human essential hypertension; and the risk of cardiovascular events is more than three times higher in salt sensitive patients (183, 270). Measurements of spontaneous arterial baroreflex sensitivity demonstrate abnormalities in the autonomic control of the cardiovascular system in association with salt sensitivity, supporting the hypothesis that salt sensitivity is at least in part neurogenically driven (44). In addition salt sensitive hypertensive subjects show an abnormal relationship between plasma catecholamines and salt intake, as they fail to suppress plasma NE levels during high sodium intake, whereas plasma NE concentrations fall significantly in salt resistant individuals on a high salt intake (29, 96). Salt sensitive patients also exhibit exaggerated pressor responses to infused NE (240).

Although abundant evidence supports SNS activation as a possible cause of salt sensitive hypertension, the exact mechanism by which salt increases SNS activity is unknown. It has been proposed that a high salt diet causes salt

retention, which modestly increases plasma NaCl concentrations, which activate brain osmoreceptors to ultimately increase SNS activity (18). Lesions of key brain areas, including the osmosensitive anteroventral third ventricular (AV3V) region, prevent a number of models of salt sensitive hypertension (34, 227). These osmosensitive neurons in the AV3V region, including the subfornical organ and organum vasculosum of the lamina terminalis, have projections to brain regions, such as the paraventricular nucleus, that can modulate SNS activity directly or via the rostral ventrolateral medulla (196, 259). AngII, and other humoral factors, appear to amplify the SNS activating actions of increased osmolality, perhaps by directly activating or sensitizing the osmosensitive neurons in the forebrain circumventricular structures (18, 196, 266). However the exact mechanism remains to be elucidated.

Measurements of whole body NE spillover, as an index of global sympathetic outflow, have been widely applied in the investigation of SNS activity in human cardiovascular diseases (59, 63). In this study we successfully employed these radioisotope dilution principles to demonstrate SNS activation in chronic AngII-salt hypertension in the rat. The advantage of this method to distinguish the dynamic processes of NE spillover and clearance is evident on analyzing the measurements obtained in the control period. Interpreting plasma NE concentrations alone during the control period may suggest a tendency towards global SNS inhibition in rats fed a high salt diet, although this was not statistically significant ($p=0.09$). However, rats fed a high salt diet during the control period

also tended (p=0.06) to have increased NE clearance. As a result NE spillover was similar independent of salt diet, indicating similar levels of global SNS outflow and the possible fallibility of interpreting plasma NE levels in isolation.

Perspectives

A high salt diet alone or AngII infusion in animals fed a normal salt diet does not increase global sympathetic outflow. However when administered in combination, the additional hypertensive response to AngII in rats fed a high salt diet is accompanied by SNS activation. Measurements of regional NE spillover and direct sympathetic nerve recordings are required to identify the regional pattern of AngII-salt mediated sympathoactivation; however regional denervation studies indicate the splanchnic bed is the critical peripheral neural target. The phenomenon of salt-sensitivity is common in human essential hypertension; however the exact mechanism remains to be elucidated. This study indicates that one possible mechanism of salt-sensitivity is SNS activation. Therefore sympathoinhibition may be a successful therapeutic strategy for salt-sensitive hypertension in humans.

CHAPTER SEVEN: REGIONAL HIND-LIMB HEMODYNAMICS AND NOREPINEPHRINE SPILLOVER IN CHRONIC ANGIOTENSIN II – SALT HYPERTENSION IN THE RAT

The splanchnic circulation appears to be the critical neural target in chronic angiotensin II (AngII) – salt hypertension in the rat (139, 141, 197). However, while celiac ganglionectomy (CGx), to selectively denervate the splanchnic circulation, substantially reduces the enhanced depressor responses to ganglion blockade in chronic AngII-salt hypertension, it does not abolish them (141). This suggests that a degree of sympathetic nervous system (SNS) activation remains in the absence of the splanchnic sympathetic system. The purpose of this study was to determine if hind-limb skeletal muscle is a target of the remaining non-splanchnic SNS activation. In addition the regional hind-limb hemodynamic profile in chronic AngII-salt hypertension was examined.

Due to the relative ease of accessibility, microneurography techniques have been widely applied to provide measures of muscle sympathetic nerve activity (MSNA) in hypertensive humans (104). In fact the most compelling evidence for SNS activation in hypertension comes from these direct MSNA recordings (3, 98, 233). AngII is mechanistically involved in these increases in MSNA as treating human hypertension with AT1 receptor antagonists significantly reduces MSNA (12) (99) (185). Therefore skeletal muscle seems a likely target of SNS activation in chronic AngII-salt hypertension. The mechanism by which skeletal muscle would likely contribute to hypertension is via an increase in vascular resistance.

Regional norepinephrine (NE) spillover measurements have been widely applied by Esler and colleagues to determine regionally selective sympathetic activation patterns in human hypertension (65, 67, 70, 72, 81). However, due to the technical difficulty, these methods have not been widely utilized in experimental animal models. In this study we developed a technique to repeatedly assess regional NE spillover to the hind-limbs in rats infused chronically with AngII. This required short-term infusion of ^{3}H-NE into the jugular vein to achieve steady-state plasma concentrations, simultaneous sampling of arterial (terminal aorta) and venous (terminal vena cava) blood from the hind-limbs and measurements of hind-limb blood flow (terminal aorta). Hind-limb NE spillover predominantly reflects sympathetic activity to skeletal muscle.

Methods

Experimental protocol

Rats were acclimatized to a 2% NaCl (n=6) or 0.4% NaCl (n=4) diet and then chronically instrumented with a perivascular flow probe on the terminal aorta and catheters positioned in the jugular vein, terminal aorta and terminal vena. Following 10 days of recovery and a 2 day control period AngII was infused subcutaneously by osmotic minipump for 14 days. Hind-limb NE spillover was measured on control day 2 and AngII infusion days 6, 10 and 14.

Animals

A total of 10 rats were used in this study, 6 were on 2% NaCl and 4 were fed 0.4% NaCl. All rats were infused with angiotensin II.

Results

Hind-limb hemodynamics

Regional hind-limb hemodynamics in response to chronic infusion of AngII in rats consuming 2% or 0.4% NaCl diets are shown in **figure 23**. MAP increased significantly for the entire duration of AngII infusion in rats fed 2% NaCl from 106 ± 2 mmHg in the control period to 155 ± 4 mmHg on day 14 of AngII infusion. Heart rate tended to decrease over the initial AngII infusion period in these rats, but this was not statistically significant. In rats fed 0.4% NaCl, MAP appeared to increase from the control period (106 ± 2 mmHg) for the duration of AngII infusion; however this was only significant on the first day of AngII (132 ± 5 mmHg) in this small group of animals. Heart rate was unaffected by AngII infusion in this group.

Hind-limb blood flow was stable during the control period and was similar in rats fed both 2% NaCl (14 ± 1 ml/min) and 0.4% NaCl (15 ± 3 ml/min). Similarly hind-limb resistance was unaffected by 2% NaCl (8 ± 1) compared to 0.4% NaCl (8 ± 2) during the control period. Hind-limb flow tended to decrease for the first week of AngII infusion in rats on 2% NaCl, although this was not statistically significant. Hind-limb flow also appeared to decrease on the first day of AngII infusion in rats

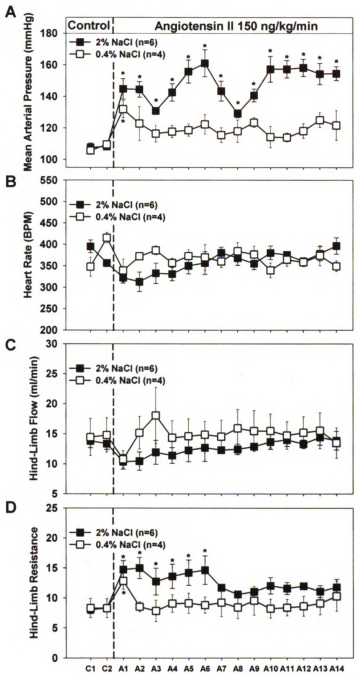


Figure 23: Regional hind-limb hemodynamics in catheterized rats in response to AngII infusion. MAP **(A)**, HR **(B)**, hind-limb blood flow **(C)** and hind-limb resistance (D) during control and AngII periods in rats fed a high salt diet. * = $p < 0.05$ compared to control period.

on 0.4% NaCl, although not significantly, but then was similar to control levels for the remainder of the AngII infusion period. In rats fed 2% NaCl, hind-limb resistance was significantly increased from control period levels for the first 6 days of AngII infusion and appeared to remain increased throughout the remainder of AngII administration, but this was not statistically significant. Hind-limb resistance was significantly increased in rats fed 0.4% NaCl for the first day of AngII infusion and then returned to baseline levels for the remainder of the experiment.

Hind-limb NE spillover

Hind-limb NE spillover during the control and AngII infusion periods is shown in **figure 24**. The average extraction fraction of NE across the hind-limb bed was 23.5% and this was unaffected by AngII infusion. Hind-limb NE spillover was similar during the control period in rats fed 0.4% NaCl (338 ± 65 pg/min) and 2% NaCl (392 ± 73 pg/min) diet. In rats fed a 0.4% NaCl diet, hind-limb NE spillover was unchanged by AngII infusion. Although hind-limb NE spillover tended to decrease on AngII infusion day 6 and increase on AngII infusion days 10 and 14 in rats fed 2% NaCl, these changes were modest in magnitude and not statistically significant.

Discussion

Hind-limb vascular resistance was significantly increased by AngII infusion in rats fed both 2% and 0.4% NaCl. However the magnitude and duration of increased

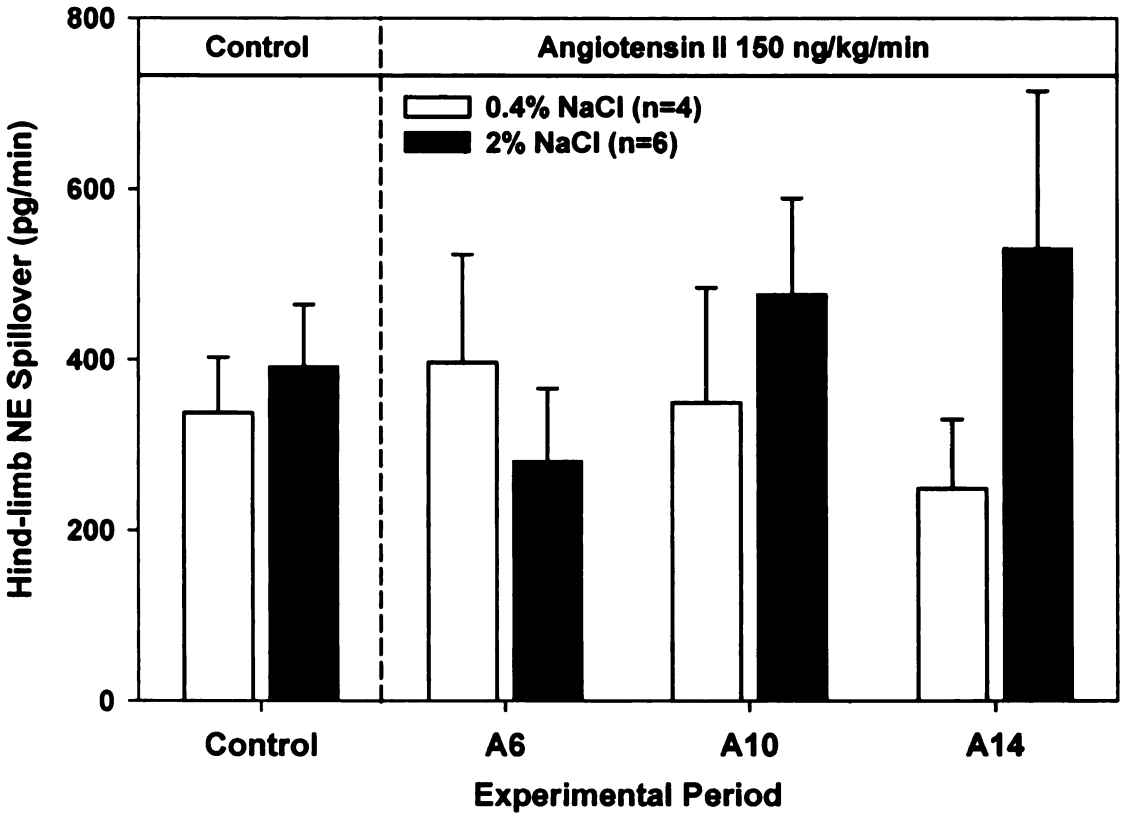


Figure 24: Hind-limb NE spillover during control and AngII infusion periods in rats fed 0.4% or 2% NaCl diet.

hind-limb resistance was significantly greater in rats fed a high salt diet, with resistance returning to control levels within 48 hours in animals fed 0.4% NaCl. This increased hind-limb vascular resistance was accompanied by a modest, but not significant, fall in hind-limb blood flow. This indicates active hind-limb vasoconstriction, which could be neural in origin, however direct constrictor actions of AngII or an autoregulatory response may be contributing. These non-neural alternative vasoconstrictor mechanisms are particular relevant in the hind-limbs, as resistance arteries from skeletal muscle produce strong myogenic responses under both normotensive and hypertensive conditions (88). This is in contrast to the relatively weak myogenic responses observed in the splanchnic bed, particularly the splenic circulation (16).

Hind-limb NE spillover measurements allowed for the neural component of the hind-limb vasoconstriction to be determined. To our knowledge this is the first report of measurements of hind-limb NE spillover in conscious, undisturbed rats. Hind-limb NE spillover was unchanged by AngII infusion in rats fed 0.4% NaCl. In rats fed 2% NaCl the largest increase in hind-limb vascular resistance was observed on AngII infusion day 6. However, hind-limb NE spillover tended to be reduced on this day, indicating that the increase in vascular resistance was not sympathetically mediated at this time point. Hind-limb NE spillover tended to be increased on AngII infusion days 10 and 14 in rats fed 2% NaCl. However this increase was not statistically significant in this small group of rats and was only

modest in magnitude. An increase of this magnitude is unlikely to have significant hemodynamic effects.

Perspectives

Chronic infusion of AngII causes a significant increase in hind-limb vascular resistance. Measurements of hind-limb NE spillover indicate that this hind-limb vasoconstriction is not sympathetically mediated and therefore must be due to non-neural mechanisms; perhaps an autoregulatory myogenic response or the direct vasoconstrictor actions of AngII. The results of this study indicate that skeletal muscle is not a major target for SNS activation in chronic AngII – salt hypertension in the rat. This is consistent with work by Esler and colleagues in hypertensive humans which showed that an AT1 receptor antagonist reduced BP but not MSNA (150). However other studies have shown a significant reduction in MSNA in hypertensive humans with chronic AT1 receptor blockade (12, 99). Therefore the effect of AngII on MSNA varies depending on the conditions under which it is studied.

CHAPTER EIGHT: THE EFFECT OF AT1 RECEPTOR DOWNREGULATION IN THE PARAVENTRICULAR NUCLEUS ON CHRONIC ANGIOTENSIN II-SALT HYPERTENSION IN THE RAT

Experimental studies suggest that systemically delivered AngII likely activates forebrain circumventricular organs, which have efferent projections to brain centers, such as the paraventricular nucleus (PVN), known to influence SNS activity (21, 43, 82, 144). It has been proposed that AngII generated locally in the PVN, to activate AT1 receptors, may be involved in activation of these central pathways (8, 36, 53, 76, 157, 159, 239, 252-255). For example, immunohistochemical localization of the immediate early gene protein product, c-fos, to identify PVN neurons activated by peripherally administered AngII, suggested that PVN neurons expressing the AT1 receptor participate importantly in the central pressor actions of circulating AngII (53, 157). In addition, whole cell patch-clamp recordings indicate that AngII excites spinally projecting PVN neurons by activating presynaptic AT1 receptors to attenuate inhibitory GABAergic synaptic inputs (157). A recent study has also shown that silencing AT1 receptor expression in the PVN, using small hairpin RNA delivered by adenovirus, attenuates the increase in arterial pressure in response to acute intravenous administration of AngII (188). Moreover, electrical stimulation of the subfornical organ, a circumventricular structure critical in mediating the central actions of AngII (43), excites PVN neurons projecting to the intermediolateral cell column in the spinal cord (the location of sympathetic pre-ganglionic neurons), which is prevented by AT1 receptor antagonism in the PVN (7).

In addition to evidence that AngII activation of AT1 receptors in the PVN contributes to the central actions of circulating AngII, a number of studies have reported the importance of PVN AT1 receptors in central hyperosmolality induced sympathoexcitation (36). Autonomic regions of the PVN are a key target of forebrain osmosensitive neurons. A major source of osmosensitive, excitatory input to the PVN is from AngII containing nerve fibers originating from cell bodies in the lamina terminalis (7, 8, 36, 74, 241). These studies suggest that the effect of central hyperosmolality to activate the SNS is dependent, in part, on activation of angiotensinergic pathways in the PVN. Infusion of hypertonic saline into the internal carotid artery increases arterial pressure and renal sympathetic nerve activity, which is significantly attenuated by microinjection of losartan, an AT1 receptor antagonist, into the PVN (36).

Combined, the above studies indicate that AngII activation of PVN AT1 receptors contributes to the autonomic responses induced by both AngII and hyperosmolality. This suggests that PVN AT1 receptors may be of particular importance to the sympathetic responses to the combination of AngII infusion in animals fed a high salt diet. In this study we tested the hypothesis that selective downregulation of AT1 receptors in the PVN will attenuate chronic AngII-salt hypertension.

Methods

Experimental protocol

Male Sprague Dawley rats were fed 2% NaCl for 7 days and a radiotelemetry transmitter was implanted to measure arterial pressure (AP) and heart rate (HR). Following 7 days of recovery and 7 days of control, an adenoviral vector ($1x10^9$ pfu/ml, 200ηl over 2 minutes) expressing AT1shRNA (AT1x) or β-galactosidase (β-gal) was microinjected bilaterally into the PVN. AngII (150 ng/kg/min) or vehicle were administered subcutaneously, beginning 10 days later, by minipump for 14 days. Body weight and 24 hr water, food and sodium intake were measured on day 10 after virus injection and on AngII or vehicle infusion days 7 and 14. The peak fall in MAP in response to hexamethonium administration (30 mg/kg IP) was measured on AngII or vehicle infusion day 14.

Animals

A total of 23 rats were used in this study and were divided into the 4 groups. β-galactosidase containing adenovirus was micro-injected bilaterally into the PVN of 11 rats. Vehicle was subsequently infused into 5 of these rats (βgal-V) and AngII was infused into the 6 remaining rats (βgal-AII). An adenoviral vector expressing AT1shRNA (AT1x) was microinjected bilaterally into the PVN of 12 additional rats. Vehicle was subsequently infused into 6 of these rats (AT1x-V) and AngII was infused into the other 6 rats (AT1x-AII).

Results

Figure 25 shows the effect of AT1 receptor knockdown in the PVN on the MAP and HR response to chronic vehicle or AngII infusion in rats fed a 2% NaCl diet.

AT1 receptor knockdown had no affect on baseline AP or HR, and cardiovascular parameters were unchanged by vehicle infusion. Contrary to my hypothesis AT1 receptor knockdown in the PVN did not affect the development of AngII-salt hypertension. On day 14 of AngII infusion MAP was 146 ± 11 mmHg in the AT1x group compared to 147 ± 7 mmHg in the β-gal treated group. HR responses to AngII infusion, showing a transient bradycardia, were also similar in the two groups.

Figure 26 displays body weight and food, water and salt intake measured 10 days after virus injection and on AngII or vehicle infusion days 7 and 14. Bilateral microinjection of AT1-shRNA did not affect body weight or food, water or salt intake during the control or vehicle infusion periods. Food and salt intake were unaffected by AngII infusion in both β-gal and AT1x groups. Water intake increased significantly on day 7 of AngII infusion in β-gal treated rats, and this increase in drinking was similar in AT1x treated rats. Similarly, AngII infusion significantly attenuated weight gain in both β-gal and AT1x rats to a similar extent.

To test if global SNS activation was affected by AT1R knockdown in the PVN, peak depressor responses to hexamethonium (30 mg/kg IP) were measured on vehicle or AngII infusion day 14 (**figure 27**). In vehicle infused rats depressor responses were similar in AT1x (-42±5 mmHg) and β-gal (-33±4 mmHg) groups.

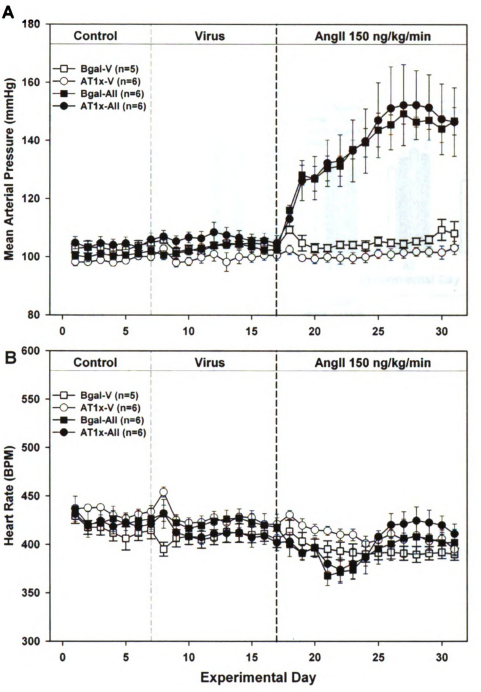


Figure 25: The effect of AT1 receptor downregulation in the PVN on MAP and HR responses to chronic infusion of AngII measured by radiotelemetry. MAP **(A)** and HR **(B)** in rats fed a high salt diet.

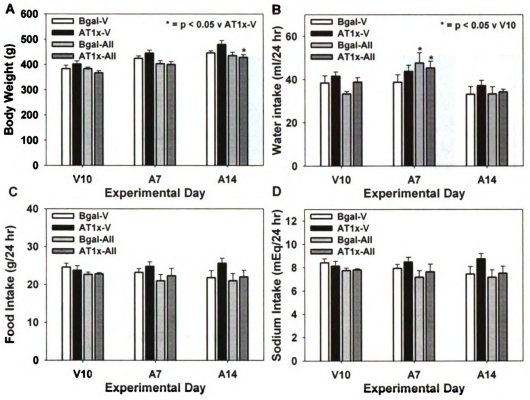


Figure 26: The effect of AT1 receptor downregulation in the PVN on body weight and food, water and salt intake during chronic infusion of AngII. Body weight **(A)**, water intake **(B)**, food intake **(C)** and salt intake **(D)** 10 days after viral microinjections (V10) and on AngII or vehicle infusion days 7 (A7) and 14 (A14) in rats fed a high salt diet * = $p < 0.05$ compared to vehicle.

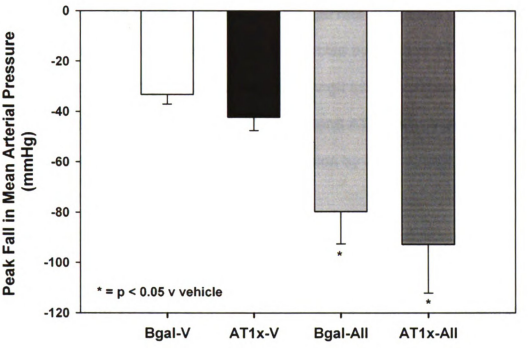


Figure 27: The effect of AT1 receptor downregulation in the PVN on responses to ganglion blockade during chronic infusion of AngII. The peak fall in mean arterial pressure after hexamethonium administration (30 mg/kg IP) on day 14 of AngII or vehicle infusion in rats microinjected with adenovirus containing β-galactosidase or AT1-shRNA. * = $p < 0.05$ compared to vehicle.

PVN AT1x treatment did not interfere with SNS activation by AngII (AT1x: -93±19 mmHg, β-gal: -80±13 mmHg).

AngII infusion caused cardiac hypertrophy in β-gal rats; again this was unaffected by AT1x (**figure 28**). Kidney weight was not affected by AngII or AT1x (**figure 28**). Western analysis (**figure 29**) showed that AngII infusion did change the expression of the AT1 receptor in the PVN. Bilateral AT1x microinjection in the PVN significantly reduced AT1 receptor expression by 41% in AngII infused rats and 29% in vehicle treated rats.

Discussion

Despite compelling evidence for a role of PVN AT1 receptors in mediating central responses to AngII and osmotic stimuli, selective knockdown of AT1 receptors in the PVN had no effect on chronic AngII-salt hypertension. In response to AngII infusion in rats fed a high salt diet, knockdown of AT1 receptors in the PVN had no effect on the arterial pressure increase, global SNS activation, dipsogenic effect or the development of cardiac hypertrophy. This leads to the conclusion that PVN AT1 receptors are not involved in mediating the central actions of chronic systemically administered AngII in the setting of a high salt intake. There is however one caveat to this conclusion. The level of AT1 receptor knockdown in the PVN in animals infused with AngII was only 42%, which was less than has been previously reported (37, 188). Knockdown of AT1 receptors by approximately 70% in forebrain circumventricular structures was sufficient to

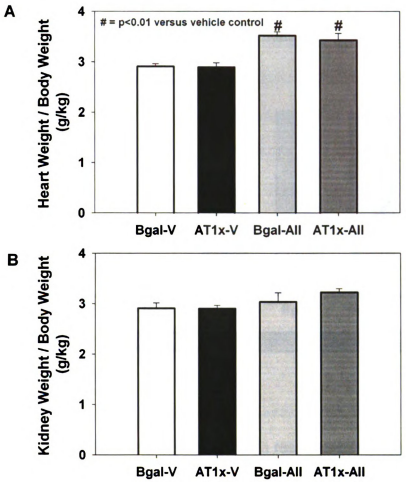


Figure 28: The effect of AT1 receptor downregulation in the PVN on the development of cardiac and renal hypertrophy in response to chronic infusion of AngII. Heart weight / body weight **(A)** and kidney weight / body weight **(B)** after 14 days of AngII or vehicle infusion in rats microinjected with adenovirus containing β-galactosidase or AT1-shRNA. # = $p < 0.05$ compared to vehicle.

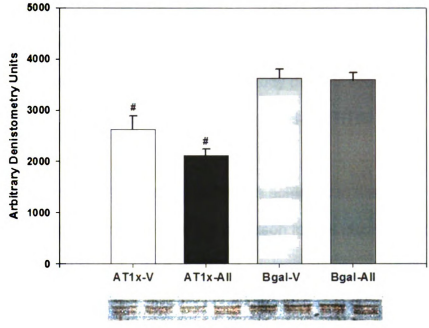


Figure 29: AT1 receptor levels in the PVN in response to chronic infusion of AngII and receptor knockdown. AT1 receptor expression in the PVN after vehicle or AngII infusion in rats microinjected with adenovirus containing β-galactosidase or AT1-shRNA. # = $p < 0.05$ compared to β-gal.

chronically exert physiological effects in normotensive mice (37). In addition, downregulation of AT1 receptors in the PVN by 52%, was sufficient to attenuate pressor responses to acute, intravenous administration of AngII (188). Therefore the less efficient receptor knockdown in this study does not completely rule out a possible role of PVN AT1 receptors in chronic AngII-salt HTN.

Providing the level of AT1 receptor knockdown achieved in this study was sufficient to exert physiological effects, the reason for the inconsistency in findings with previously published studies is unclear. However one likely explanation is that a significantly different model system was exploited in the current study compared to previous studies examining the role of PVN AT1 receptors. The most notable distinction is the chronicity and magnitude of the applied physiological stimulus. For example, Chen and Toney examined the effects of AT1 receptor antagonism in the PVN on hemodynamic and sympathetic responses to acute and marked increases central osmolality by intra-carotid delivery of hypertonic saline (36). In the current study the effects of a more sustained and modest osmotic load administered orally was examined. To further emphasize the difference in these models, Chen and Toney recorded significant elevations in renal SNS activity (36), whereas renal SNS activity is documented to decrease in this model of chronic AngII-salt hypertension (141). Northcott and coworkers reported the importance of PVN AT1 receptors in the pressor response to acute intravenous administration of AngII (188). However the current study explored the role of PVN AT1 receptors in response to a

chronic administration of a low dose of AngII that only increases plasma AngII levels two-fold and result in plasma concentrations within the pathophysiological range (267). Whereas the evidence for a role of PVN AT1 receptors in acute hemodynamic and sympathetic responses is solid, their role in a chronic physiological setting has not been established.

Perspectives

The results of this study do not exclude the possibility that the classically proposed anatomical pathway mediating the central actions of AngII are functioning. It is still possible that circulating AngII activates forebrain circumventricular organs, which have efferent projections to osmotically sensitized brain centers, like the PVN, to increase SNS activity (21, 43, 82, 144). The current results merely argue against AT1 receptor activation in the PVN as an activating pathway. The PVN receives a diverse array of excitatory inputs from the lamina terminalis in addition to AngII, including glutaminergic (75), adrenergic (117) and cholinergic (216). It is likely that one or more of these transmitters are involved in activating the central circuitry in chronic AngII-salt hypertension, although this remains to be proven.

CHAPTER NINE: INFLAMMATORY MEDIATORS OF SYMPATHETIC ACTIVATION IN CHRONIC ANGIOTENSIN II HYPERTENSION

Recent findings indicate that pro-inflammatory actions of AngII, including an increased generation of reactive oxygen species and various inflammatory factors, are an intermediate step in most of the various pressor mechanisms stimulated by AngII (155, 214, 277, 296). The purpose of this study was two-fold. Firstly, to determine if increased systemic production of inflammatory cytokines could mediate sympathoexcitation in AngII hypertension. Secondly, to determine the role of cyclooxygenase derived inflammatory products in AngII hypertension and associated sympathoactivation.

Inflammatory cytokines

In the brain, AngII may stimulate local production of pro-inflammatory cytokines which act as signaling molecules to produce sympathoexcitation (296). Another possibility is that circulating AngII could increase systemic production of pro-inflammatory cytokines (52, 86, 171, 228), which would then travel to the brain to affect autonomic regulatory pathways. Increased release of cytokines into the bloodstream with subsequent actions on the brain, seems to explain, for example, some neurohormonal responses to heart failure (73), including sympathoexcitation (295).

The cytokines measured in these experiments, to determine if increased systemic production of inflammatory cytokines could mediate sympathoexcitation

in AngII hypertension, were selected based on published evidence that they are important pro-inflammatory biomarkers and that their synthesis and release are affected by AngII. For example, blocking AngII is reported to reduce circulating TNF-α levels (52, 171, 228) and, more conclusively, treatment with a TNF-α antagonist was shown to slow the development of ANGII hypertension in rats on a high salt diet (60). Furthermore, exogenous TNF-α was shown to cause sympathoexcitation in rats (295). Likewise, AngII receptor blockade is reported to decrease circulating IL-6 concentrations in hypertensive patients (171). But more importantly, chronic infusion of AngII in mice increased blood-borne IL-6 concentrations, and AngII hypertension development was attenuated in IL-6 knockout animals (155). Circulating levels of IL-1β are reported to decrease in response to AngII receptor blockade in patients with essential hypertension (158) and in SHR rats (228), although there are no studies that provide a direct link between IL-1β and AngII hypertension. Finally, we studied MCP-1 because AngII hypertension is associated with enhanced expression of this important pro-inflammatory marker in both blood vessels (124) and kidney (200).

Vasoactive prostaglandins

Cyclooxygenase derived prostanoids have been implicated in the pathogenesis of chronic AngII hypertension in the rat. AngII signaling, through AT1 receptors, activates phopholipase A_2 leading to release of arachidonic acid which is subsequently metabolized by cyclooxygenase to generate vasoactive

prostaglandins, including thromboxane A_2 (TxA_2) which activates vasoconstrictor thromboxane prostanoid (TP) receptors.

Pharmacologic blockade or genetic deletion of COX-1 in mice abolishes the pressor effect to AngII (211). In addition TP receptor knock-out mice have an attenuated increase in arterial pressure in response to chronic slow pressor infusion of AngII (133). Of particular interest is the finding that TP receptor antagonism dramatically reduced blood pressure in chronic AngII hypertensive male Sprague-Dawley rats drinking salt water (182). A recent study of AngII dependent, salt-sensitive, two-kidney, one-clip Goldblatt hypertension in the rat, found arterial pressure was lowered to a similar extent by AT1 receptor antagonism (24 mmHg) and both nonselective (25 mmHg) and COX-1 (28 mmHg) selective, cyclooxygenase blockade (271). These studies, among others, suggest an important role for vasoactive prostaglandins in contributing to the blood pressure raising actions of AngII.

Most investigators have attributed the effects of prostaglandins in AngII hypertension to their direct actions to vasoconstrict blood vessels, including renal vessels (163, 182, 278), or via actions in the kidney such as, activation of renal tubuloglomerular feedback responses (272, 273) and enhanced renal NaCl retention (274). However there is evidence to indicate that the effects of prostaglandins may be neurogenic in origin by potentiating the central actions of AngII. For example, hypertension caused by chronic infusion of TP receptor

agonists is associated with enhanced depressor responses to hexamethonium and prazosin administration, indicating global SNS activation (93). Central TP receptors also contribute to the dipsogenic response to AngII infusion (142) and activation of brain TP receptors releases AVP (279). Therefore we determined the role of cyclooxygenase derived inflammatory products in our model of AngII hypertension and the associated sympathoactivation.

Experimental protocol

Circulating levels of pro-inflammatory cytokines

Rats were acclimatized to a 2% NaCl for 7 days then instrumented with an arterial and venous catheter. Following a 3 day recovery period and a 3 day control period, an AngII or saline vehicle filled osmotic minipump was implanted subcutaneously, to deliver AngII (150ng/kg/min) or vehicle for 14 days. Arterial pressure was measured daily for the duration of the experiment. Blood samples (0.5 ml) were obtained from the venous catheter on control day 2 and AngII or vehicle infusion days 1, 3, 5, 7, 9, 11 and 13 for the measurement of monocyte chemoattractant protein 1 (MCP-1), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α).

Effect of COX inhibition on chronic AngII hypertension

Rats were fed a normal (0.4 % NaCl) or high salt (2% NaCl) diet for seven days and then a radiotelemetry transmitter was implanted to remotely monitor arterial pressure and heart rate. Following 7 days of recovery and a 4 day control period,

saline vehicle or the potent non-selective COX inhibitor ketoprofen (2 mg/kg) were injected subcutaneously once daily for the remainder of the study. After 4 days of COX inhibition or vehicle injection, AngII (150 ng/kg/min) was delivered subcutaneously by osmotic minipump for 14 days. A separate group of rats received only injections of COX inhibitor for the duration of the study and were not infused with AngII.

Effect of COX inhibition on AngII-salt mediated SNS activation

To investigate the effect of cyclooxygenase inhibition on SNS activation in AngII-salt HTN an additional group of animals where instrumented to allow measurements of whole body NE spillover. Rats were acclimatized to a 2% NaCl diet for 7 days, and then instrumented with an arterial and venous catheter. Following 5 days of recovery and a 3 day control period, AngII (150ng/kg/min) was delivered subcutaneously by minipump for 14 days. Ketoprofen (2 mg/kg IV) was administered daily to one group for the entire duration of the experiment. Endogenous plasma NE levels and total body NE spillover and clearance were determined on control day 3 and AngII infusion days 7 (A7) and 14 (A14). The peak fall in MAP in response to hexamethonium administration was measured on day 14 of AngII infusion.

Results

Circulating levels of proinflammatory cytokines

Hemodynamics

There were no significant differences in mean arterial pressure or heart rate in the two groups of rats during the control period (**figure 30**). Infusion of AngII resulted in an immediate and significant increase in mean arterial pressure that was sustained over the entire 14-day infusion period (105 ± 3 mmHg during the control period versus 133 ± 6 on infusion day 14). Arterial pressure did not change significantly in rats that received vehicle (105 ± 1 mmHg during the control period versus 100 ± 3 mmHg on infusion day 14). No significant alterations in heart rate were observed during the infusion period in either group.

Serum cytokines

Serum concentrations of TNF-α, IL-6, MCP-1 and IL-1β did not differ between the two groups of rats during the control period (**Figure 31**). In response to infusion of AngII there were no significant changes in plasma levels of TNF-α or IL-6 when compared to control period levels. There was a significant and sustained decline in plasma MCP-1 starting on day 3 of infusion. Levels of IL-1β generally were not changed by AngII infusion, but were significantly lower on days 1, 11 and 13 of the infusion period. Vehicle administration did not significantly affect serum concentrations of any cytokine.

Effect of cyclooxygenase inhibition on chronic AngII hypertension

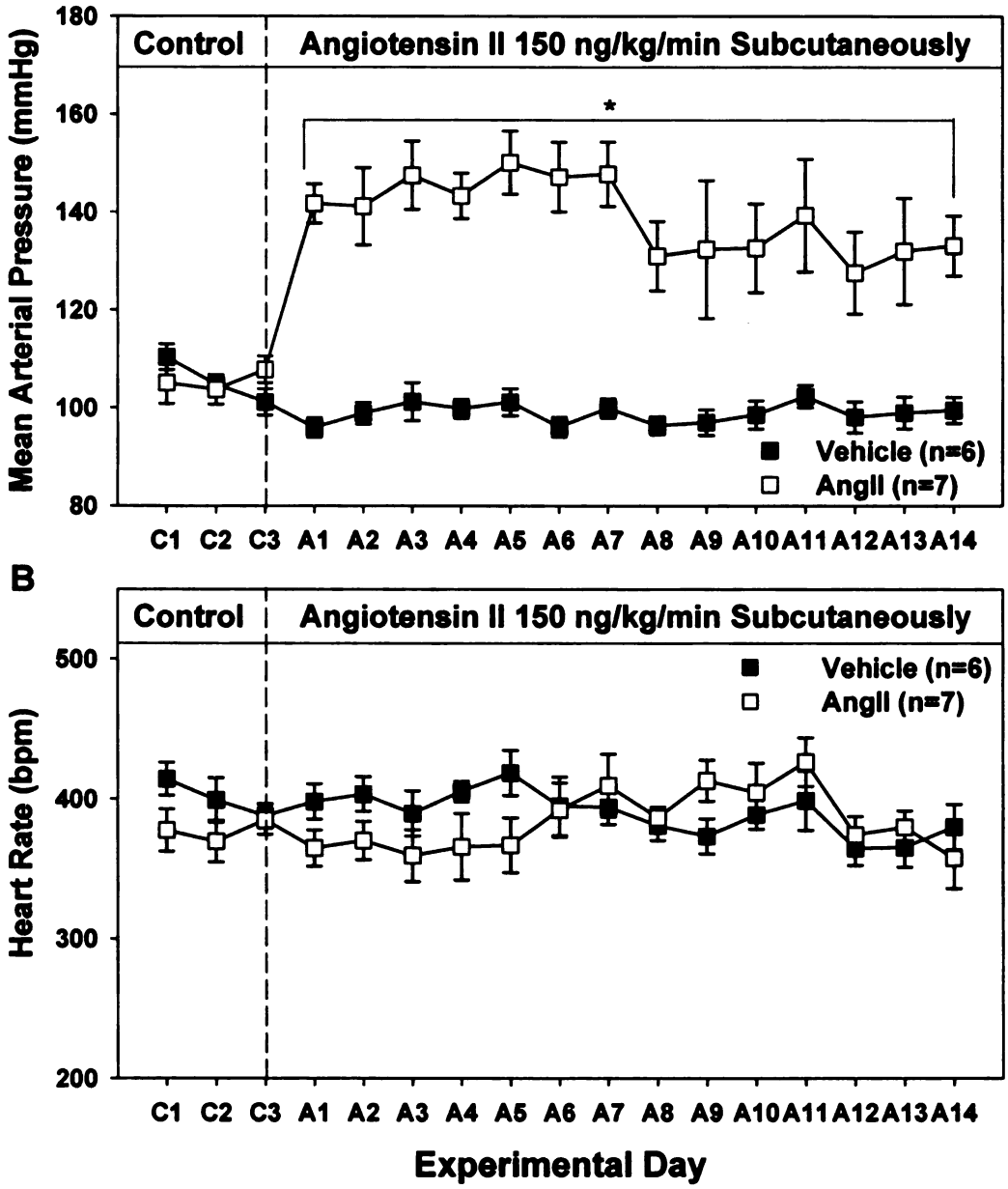


Figure 30: MAP and HR responses to chronic infusion of AngII in catheterized rats used for cytokine measurements. MAP **(A)** and HR **(B)** in rats fed 2% NaCl. *Significant difference ($P<0.05$) between specific AngII infusion day and control period average (C1, C2 and C3).

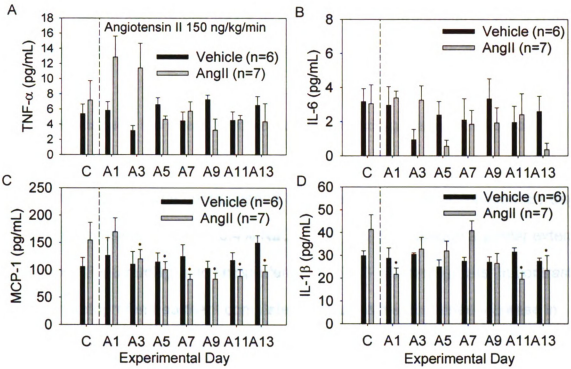


Figure 31: Temporal profile of serum cytokine levels in rats infused with AngII and fed a high salt diet. Serum concentrations of TNF-α **(A)**, IL-6 **(B)**, MCP-1 **(C)** and IL-1β **(D)** during the control period and in response to chronic subcutaneous infusion of AngII (150 ng/kg/min) or saline vehicle in rats fed 2% NaCl. *Significant difference ($P<0.05$) between specific AngII infusion day and control period.

Figure 32 shows MAP in normotensive rats fed 0.4% or 2% NaCl that were treated daily with the non-selective cyclooxygenase inhibitor ketoprofen. Chronic administration of ketoprofen had no affect on MAP for the duration of the 3 week treatment period in normotensive rats fed either 0.4 % or 2% NaCl or high diet.

Figure 33 displays the effect of non-selective cyclooxygenase inhibition on chronic AngII hypertension. In rats fed 0.4% NaCl, ketoprofen treatment had no effect on MAP during the control period or the response to AngII infusion. By day 14 of AngII infusion in rats fed 0.4% NaCl, MAP had increased to a similar extent in control (18 ± 5 mmHg) and ketoprofen treated (13 ± 5 mmHg) rats. In contrast, however, ketoprofen treatment completely abolished the sustained increase in MAP in response to AngII infusion in rats fed 2% NaCl. Although the increase in MAP over the first 5 days of AngII infusion was similar in control (22 ± 2 mmHg) and ketoprofen treated (20 ± 5 mmHg) rats fed 2% NaCl, by day 14 of AngII infusion MAP had increased significantly greater in control rats (36 ± 12 mmHg) compared to ketoprofen treated rats (2 ± 1 mmHg).

Effect of COX inhibition on AngII-salt mediated SNS activation

Figure 34 shows the MAP response, measured by exteriorized catheter, to chronic AngII infusion in rats fed 2% NaCl treated daily with the non-selective cyclooxygenase inhibitor ketoprofen or vehicle. Consistent with the previous findings when MAP was measured by radiotelemetry, the increase in MAP over the first 4 days of AngII infusion was similar in control (38 ± 4 mmHg) and

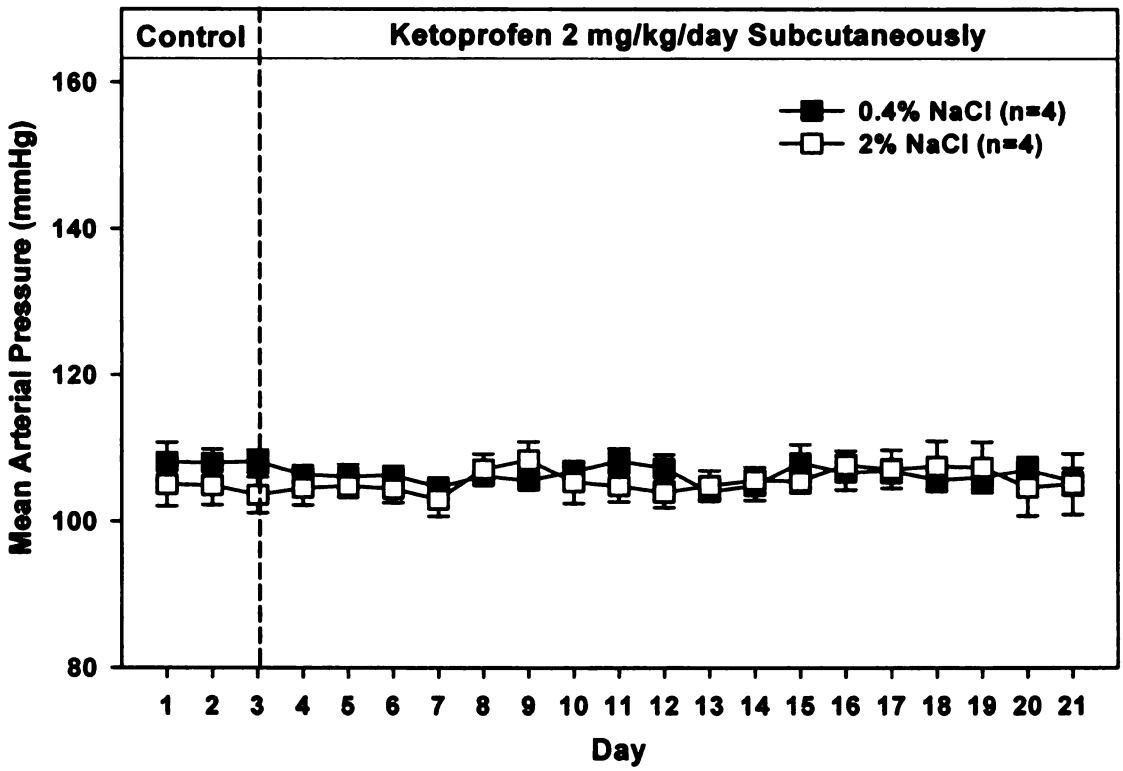


Figure 32: The effect of chronic cyclooxygenase inhibition on MAP in normotensive rats measured by radiotelemetry. Rats were fed 0.4% or 2% NaCl and treated daily with the non-selective cyclooxygenase inhibitor ketoprofen.

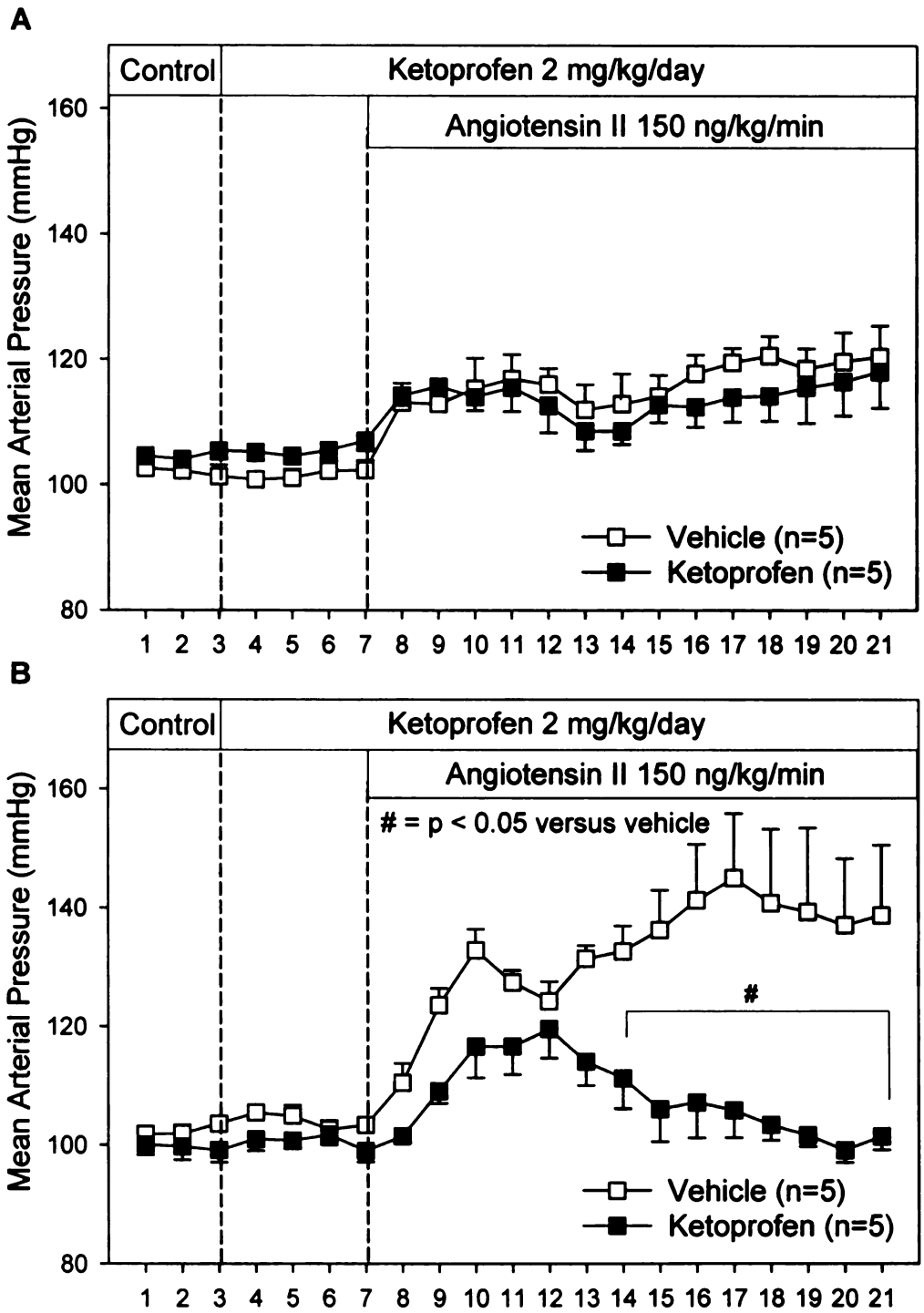


Figure 33: The effect of chronic cyclooxygenase inhibition on MAP response to AngII measured by radiotelemetry MAP in rats fed 0.4% **(A)** or 2% **(B)** NaCl.

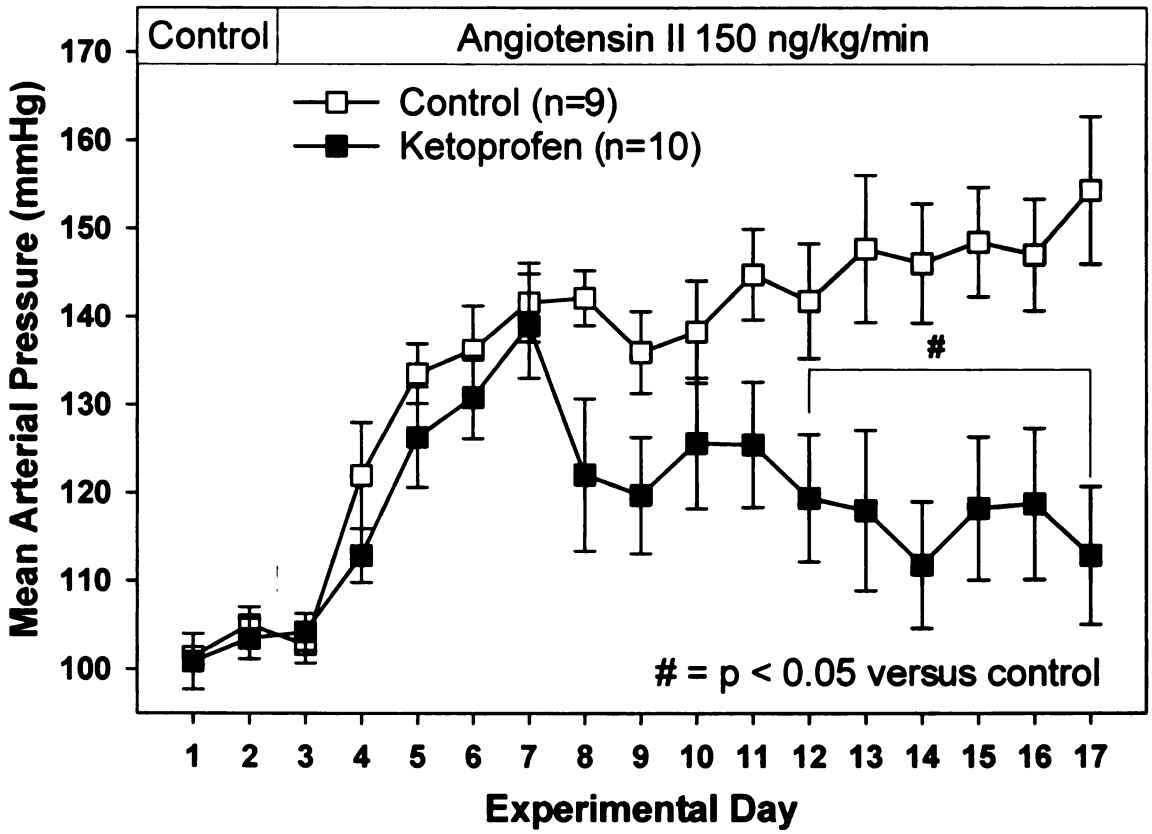


Figure 34: The effect of chronic cyclooxygenase inhibition on MAP response to AngII in catheterized rats fed a high salt diet. Rats were treated daily with the non-selective cyclooxygenase inhibitor ketoprofen or vehicle.

ketoprofen treated (36 ± 6 mmHg) rats fed 2% NaCl, however, by day 14 of AngII infusion MAP had increased significantly greater in control rats (51 ± 9 mmHg) compared to ketoprofen treated rats (10 ± 7 mmHg).

The peak fall in MAP in response to hexamethonium administration **(figure 35)** on AngII infusion day 14, in rats fed 2% NaCl, tended to be less in ketoprofen treated rats (- 63 ± 8 mmHg) than control rats (- 91 ± 14 mmHg), however this was not statistically significant (p=0.11). **Figure 36** shows the effect of ketoprofen treatment on plasma NE, NE clearance and NE spillover on control day 3 and AngII infusion days 7 and 14 in control and ketoprofen treated rats fed 2% NaCl. The global SNS activation caused by AngII infusion, as indicated by significant elevations in plasma NE and whole body NE spillover, was largely, but not completely, prevented by non-selective cyclooxygenase inhibition.

Discussion

Circulating levels of pro-inflammatory cytokines

It is now established that AngII exerts a physiologically significant pro-inflammatory effect that contributes to numerous cardiovascular diseases (48, 79). Furthermore there is extensive evidence that these pro-inflammatory actions play a part in the development of AngII hypertension (155, 214, 277, 296). AngII appears to produce inflammatory responses predominantly in a localized manner in target tissues such as blood vessels (214), kidney (277) and brain (296). Under certain conditions, however, circulating levels of various pro-

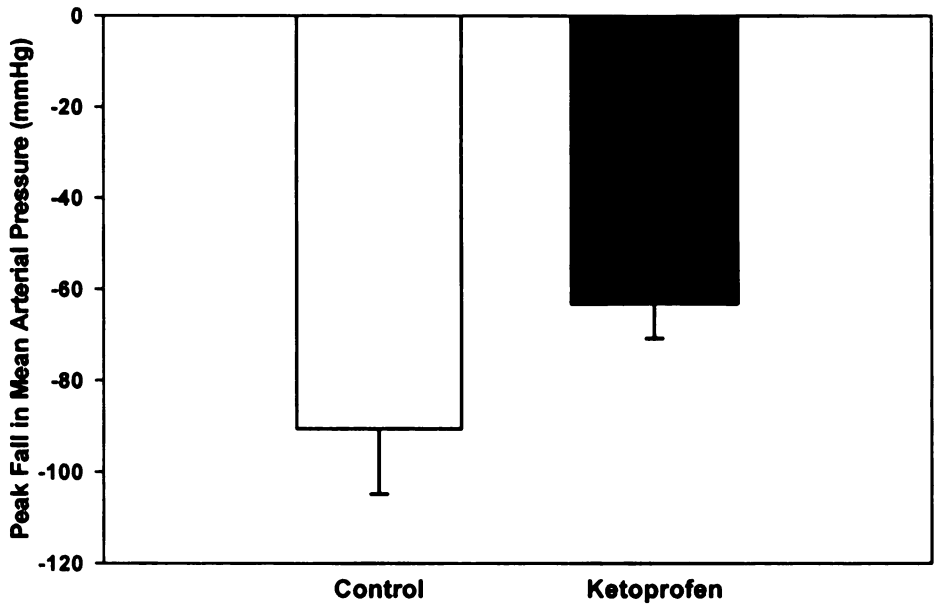


Figure 35: The effect of chronic cyclooxygenase inhibition on the depressor response to ganglion blockade in rats infused with AngII and fed a high salt diet Peak fall in MAP in response to hexamethonium administration (30 mg/kg iv) on day 14 of AngII infusion in rats fed 2% NaCl treated daily with the non-selective cyclooxygenase inhibitor ketoprofen or vehicle.

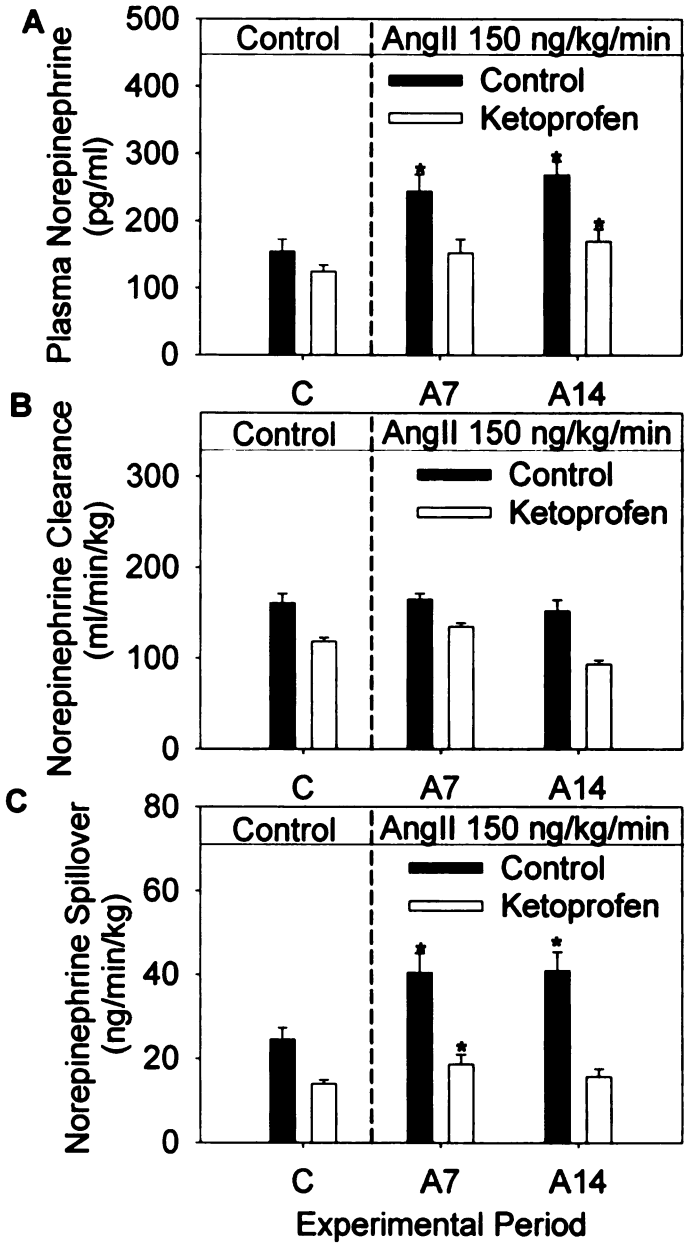


Figure 36: The effect of chronic cyclooxygenase inhibition on plasma NE, NE clearance and NE spillover in rats infused with AngII and fed a high salt diet Plasma NE **(A)**, NE clearance **(B)** and NE spillover **(C)**. * = $p < 0.05$ versus control.

Inflammatory markers may be increased as well (52, 86, 155, 171, 228). Increased blood concentrations of cytokines act as a signal to the brain to induce fever in systemic inflammation (220), and recently a similar mechanism has been proposed to operate in heart failure secondary to myocardial infarction (73), with one consequence being sympathoexcitation (295). Therefore, we hypothesized that this mechanism also might contribute to sympathetic activation in AngII hypertension. If so, then blood levels of key pro-inflammatory cytokines would be expected to increase prior to, or in concert with, the development of hypertension in rats receiving chronic AngII infusion. Our results did not confirm that expectation: circulating concentrations of the cytokines either decreased during AngII infusion or did not change. We conclude that increased sympathetic activity in AngII hypertension is not likely to be driven by circulating proinflammatory cytokines.

Neurogenic mechanisms participate in the development of hypertension in the model studied here—chronic AngII infusion in rats on a high salt diet (19, 194, 231). But sympathetic activity was not evaluated in these experiments. Rather the emphasis was on identifying a possible intermediate signaling pathway for sympathoexcitation linked to blood-borne cytokines. Inactivation of circumventricular organs (CVOs) attenuates hypertension in AngII hypertension (43, 82, 296). This is an important feature for the purpose of this study because a high density of binding sites for cytokines is found in these structures that lack the normal blood-brain barrier (220). It should be noted, however, that additional

mechanisms—not involving the CVOs—have been proposed to explain how circulating cytokines could affect brain cells without traversing the blood-brain barrier (73, 295).

Despite studying this array of multiple cytokines with diverse but overlapping functions in inflammation (79), the results were quite homogeneous: circulating concentrations measured at numerous time points during the experiment were either decreased or unchanged by AngII infusion. This finding appears to rule out circulating cytokines as an intermediate signal to the brain to cause sympathoexcitation in AngII hypertension. However, some receptors for cytokines in brain CVOs can be up-regulated during systemic inflammation (220). If this occurs during chronic AngII infusion, higher blood concentrations of key cytokines may not be required to elicit signaling within the brain. This possibility requires further investigation.

Vasoactive prostaglandins

Non-selective cyclooxygenase inhibition had no effect on chronic AngII hypertension in animals fed a normal salt diet; however, ketoprofen completely abolished the sustained increase in arterial pressure and largely prevented SNS activation in response to chronic AngII infusion in rats fed a high salt diet. This indicates that cyclooxygenase derived prostaglandins are contributing, at least in part, to chronic AngII-salt hypertension by potentiating the SNS activating actions of AngII. The exact nature of the cyclooxygenase product responsible for

mediating these actions of AngII is unclear. TP receptor antagonism has consistently been reported to lower BP in chronic AngII hypertension (182, 280-282), whereas the effects of inhibiting the synthesis of TxA_2 have been inconsistent (182, 281, 282). These results are most consistent with TP receptor activation in chronic AngII hypertension by TxA_2 or more likely a prostaglandin endoperoxide that also activates TP receptors. Nasjletti has proposed that PGH_2 is the major COX-dependent TP receptor activator in AngII induced hypertension (182).

There are striking similarities between the hypertension that develops in response to pharmacologic activation of TP receptors and that of the classic slow pressor AngII hypertension, indicating that TP receptor activation may be responsible for many of the pro-hypertensive actions of AngII (134, 271). Hypertension that develops in response to intravenous administration of a TP receptor agonist is prevented by intracerebroventricular delivery of a TP receptor antagonist (279), suggesting the predominant mechanism by which vasoactive prostaglandins chronically increase arterial pressure is neurogenic in nature.

We have shown that selective removal of splanchnic SNS innervation significantly attenuates chronic AngII-salt hypertension (141), likely indicating that splanchnic SNS activity is increased in this model. Intriguingly, direct recordings of splanchnic nerve activity have documented increased splanchnic SNS activity in chronic AngII hypertension (167). This was associated with significant

elevations in urinary prostaglandins and the authors concluded that the increase in splanchnic SNS tone is possibly mediated by prostaglandins (167). This provides a direct link between prostaglandins mediating splanchnic SNS activation and chronic AngII hypertension.

Perspectives

This study shows that in rats fed a high salt diet, chronic AngII infusion, activates the SNS to increase arterial pressure by a cyclooxygenase dependent pathway. Therefore cyclooxygenase inhibition may represent a successful therapeutic strategy in treating AngII dependent hypertension. In fact, non-selective cyclooxygenase inhibitors, such as aspirin and indomethacin, significantly reduce blood pressure in human renovascular hypertension (122); a type of hypertension characterized by hyperreninemia and elevated plasma levels of AngII.

CHAPTER TEN: DISCUSSION

Although progress has been made in proving that SNS overactivity is involved in the pathogenesis of HTN, identifying the specific mechanisms of neurogenic HTN requires further understanding. Neither the critical CNS networks nor the peripheral SNS efferents have been unequivocally defined. This is best put in context by Guyenet in a recent review published in *Nature Reviews Neuroscience* on the sympathetic control of BP (104), where he states "although evidence that the brain regulates the 24-h average BP and contributes to the hypertensive process is very persuasive, the mechanisms are not well understood". Further he indicates that "the sympathetic efferents that innervate the kidneys are commonly presented as the only ones that are capable of influencing the 24-h average BP" (104). The work in this thesis challenges that notion and documents an important role of the splanchnic SNS in mediating long-term changes in BP. This is particularly timely given that Eisenhofer noted in his recent review on sympathetic nerve function; "due to difficulties of accessibility, the function of the sympathetic nerves innervating the splanchnic organs remains largely hidden from view" (59).

10.1. Hemodynamic mechanism of AngII – salt HTN in rats

The key findings of this thesis are summarized into a mechanistic schema depicted in **figure 37.** Chronic infusion of AngII appears to cause

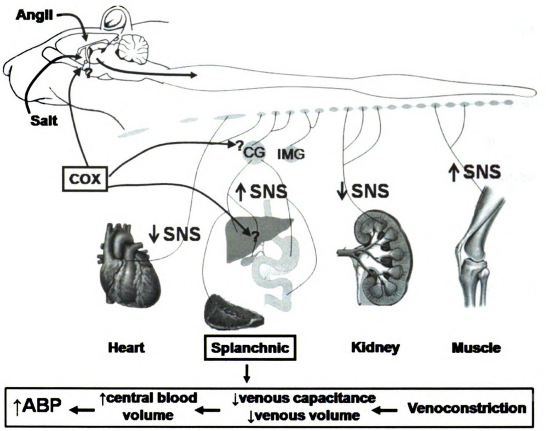


Figure 37: Schematic of conclusions. Chronic infusion of angiotensin II causes sustained elevations in arterial pressure (AP) in rats fed a high salt diet, in part, by increasing sympathetic nervous system (SNS) activity, via a cyclooxygenase (COX) dependent pathway, to the high-capacitance splanchnic organs. Sympathetically mediated venoconstriction reduces venous capacitance and venous blood volume, causing a translocation of blood to the less compliant arterial circulation. This translocation, in the presence of a defect in renal excretion, ultimately increases AP.

sustained elevations in AP in rats fed a high salt diet, in part, by increasing SNS activity to the high-capacitance splanchnic organs. Sympathetically mediated venoconstriction reduces venous capacitance and venous blood volume, causing a translocation of blood to the less compliant arterial circulation. This translocation of blood increases central blood volume and ultimately increases AP. Cyclooxygenase derived inflammatory products appear to be critical intermediates in the pathway that mediates SNS activation by AngII in animals fed a high salt diet.

These conclusions are conceptually supported by previous studies showing that increases in splanchnic SNS activity cause a translocation of blood towards the heart, increasing cardiac diastolic filling and cardiac output (101, 102). In fact a 1946 study definitively showed that electrical stimulation of the splanchnic nerve in dogs, for as long as 41 days, causes sustained hypertension without detectable changes in renal blood flow, glomerular filtration rate, filtration fraction or renal function (151). The author's concluded that the persistent elevation in BP was a result of direct vasoconstriction of the splanchnic bed (151). This provides proof-of-concept support for a principal finding of my studies that hemodynamic changes in the splanchnic bed caused by sympathetic nerve activity are a mechanism of HTN.

10.2. Alternate hemodynamic mechanisms of AngII HTN in rats

The major limitation of the mechanism proposed above is that it relies on a translocation of blood from the venous system to the less compliant arterial circulation. This translocation is based on the finding of a massive reduction in venous capacitance, presumably resulting in a reduction in venous volume, without detectable changes in total vascular volume in response to chronic infusion of AngII in rats fed a high salt diet. It is likely that at least some of this volume is translocated to the arterial circulation. A very small increase in arterial volume will significantly increase BP. For example, it has been estimated that an increase in arterial volume of 1 ml will increase BP 60 mmHg (288). However, my studies have not documented this proposed arterial translocation of blood and it is possible that other vascular beds such as the pulmonary circulation or the heart are accommodating this volume. This gives rise to the possibility that mechanisms other than an arterial translocation of blood may be contributing to the pathogenesis of chronic AngII-salt HTN.

The quantitative contribution of venoconstriction to the increase in BP in response to chronic AngII infusion in rats fed a high salt diet is unclear. The evidence for reduced vascular capacitance is unequivocal; however the question as to the causal relationship between venoconstriction and the HTN remains. Selective removal of the splanchnic SNS prevented the venoconstriction and attenuated the increase in BP in response to AngII infusion in rats on a high salt diet. However this surgical procedure is not selective for venous innervation as the splanchnic arterial sympathetic nerves are also removed. Venoconstriction is

classically thought to support AP via CO related mechanisms. Osborn's laboratory failed to identify increases in CO and found that the predominant hemodynamic mechanism of chronic AngII-salt HTN was an increase in TPR (197). Although it is quite possible that a transient and modest increase in CO as a result of a venous to arterial translocation of a small volume of blood is beyond the resolution of the measurement technique, this study along with my work is also consistent with an alternate mechanism of AngII-salt HTN; that is sympathetically mediated constriction of splanchnic resistance vessels. I have shown that splanchnic vascular resistance significantly increases in response to AngII infusion in rats fed a high salt diet and Osborn's group found that CGx abolishes the chronic vasoconstrictor actions of AngII in rats on high salt (197). Together these results indicate that splanchnic resistance vessels make an important hemodynamic contribution to chronic AngII-salt hypertension. It is likely that sympathetically mediated vasoconstriction of both splanchnic resistance and capacitance vessels contribute to the chronic elevations in AP in AngII-salt HTN, although the relative contribution of each is unknown.

An alternative explanation to the effect of CGx to attenuate chronic AngII-salt HTN is via denervation of the adrenal medulla and prevention of catecholamine release into the circulation. CGx suppresses the sympathoadrenal response to insulin-induced hypoglycemia (90) indicating the celiac ganglion is required for sympathetic responses of the adrenal gland. AngII stimulates the release of both NE and EPI from the adrenal medulla and evidence for this has been reviewed

by Reid (217). The stimulatory effect of AngII on catecholamine release appears in part to involve a direct action of AngII on adrenal medulla AT1 receptors (23). In addition AngII enhances adrenal catecholamine secretion in response to splanchnic nerve stimulation (169). Therefore it is possible that CGx attenuated AngII-salt HTN by impairing adrenal medulla catecholamine secretion. Surgical adrenal demedullation is required to test this hypothesis, however adrenalectomy did not affect the pressor response to AngII infusion in dogs (55, 205).

It is also possible to envision a scenario in which the splanchnic SNS is critical to the development of AngII-salt hypertension where splanchnic SNS outflow is not increased. The effect of AngII to facilitate neurotransmission at the sympathetic nerve terminal has been extensively studied (217). Experimental evidence indicates that AngII increases the bioavailability of NE in the junctional cleft by enhancing the release and decreasing the reuptake of NE (203). These effects are dependent on a presynaptic action of AngII on AT1 receptors (204, 246). The ability of AngII to facilitate NE release is most pronounced at low, physiological frequencies of nerve firing (203). AngII also increases the biosynthesis of NE by increasing the expression of tyrosine hydroxylase (221), and post-synaptic vasoconstrictor responses to NE are enhanced by AngII (217). The splanchnic circulation is densely innervated by the SNS and denervation of this bed removes a major site for AngII to facilitate noradrenergic neurotransmission. In this scenario the splanchnic SNS is required for the actions of AngII, but overt SNS activation is not necessary.

Clearly non-neural mechanisms also play an important role in the pathogenesis of chronic AngII HTN. I failed to identify a significant role of the SNS in the increase in BP in response to infusion of AngII in animals fed a normal salt diet, obviously indicating non-neural mechanisms predominate. CGx attenuated AngII-salt HTN, but did not abolish it. In fact, approximately 45% of the increase in BP in response to AngII infusion in rats fed a high salt diet remained after CGx, indicating non-neural mechanisms also play a significant role in AngII-salt HTN. These non-sympathetic mechanisms likely include direct renal and vascular effects of AngII. The role of the renal effects of AngII in HTN has been extensively investigated by Guyton's group. AngII exhibits powerful direct antinatriuretic effects (291). Although I did not document blood volume expansion in my studies, it is possible that subtle volume related changes occurred beyond the resolution of my measurements. AngII also has pleotrophic vascular effects that can contribute to the hypertensive process. Not only is AngII a potent direct vasoconstrictor but it also induces endothelial cell dysfunction and vascular remodeling via effects on cell growth and apoptosis, inflammation and fibrosis (232). Activation of major intracellular signaling cascades can produce structural and functional vascular changes that contribute to chronic elevations in BP (260).

Realistically, HTN caused by chronic infusion of AngII is multi-factorial and likely involves a complex interaction between the neural, renal and vascular actions of AngII. The neural component is particularly important in the setting of a high salt diet and requires the presence of the splanchnic SNS. Sympathetic innervation to

non-splanchnic beds does not appear to make a significant contribution to the hemodynamics of chronic AngII-salt HTN.

10.3. Cyclooxygenase dependent sympathetic activation

One of the more intriguing findings of this thesis work is the apparently critical role of cyclooxygenase derived products in mediating AngII induced SNS activation in rats fed a high salt diet. Elucidating the exact nature and tissue location of the vasoactive prostanoid responsible for the sympathetic response to AngII could prove productive in identifying a novel therapeutic strategy for the treatment of salt-sensitive neurogenic HTN. Although the role of cyclooxygenase products in mediating sympathetic responses in HTN is yet to be fully established, a case description of two siblings with renin-dependent HTN provides preliminary support of a critical involvement of prostaglandins in sympathetic overactivity in human HTN (54). These patients exhibited a dramatic fall in BP in response to the non-selective β-adrenergic receptor antagonist propranolol, which documents a clear increase in sympathetic tone (54). More importantly, oral administration of the non-selective cyclooxygenase inhibitor indomethacin also significantly decreased BP in these subjects (54). This suggests that SNS activation and the sustained elevation in BP in a human form of AngII dependent HTN may be driven by prostaglandins.

Further insight into prostanoid mediated sympathoactivation is apparent by examining other systems. Recently, cyclooxygenase antagonism has been

shown to inhibit exercise induced activation of muscle sympathetic nerves (46), implicating prostanoids as important intermediates in reflex sympathetic responses. Furthermore, Felder has elucidated a critical role for prostaglandin (PG) dependent mechanisms, at the level of the hypothalamus, in stimulating sympathetic activity (295). For example, direct injection of PGE(2) into the lateral ventricle or PVN significantly increases sympathetic drive; and injecting cyclooxygenase antagonists into the lateral ventricle attenuates sympathetic and BP responses to systemically administered cytokines (295). Brain centers implicated in eliciting the central pressor actions of AngII, such as the SFO, AP, PVN and RVLM robustly express prostanoid receptors (294). The exact source of prostanoids that activate these receptors to increase sympathetic activity is unknown, but it has been proposed that cerebral microvessels produce large quantities of soluble PGs that readily diffuse across the blood-brain-barrier to act on neurons (22, 61, 295). This pathway is best established for the actions of inflammatory cytokines. However, AngII has been shown to stimulate vasoactive prostanoid production by human cerebromicrovascular endothelium by activating phopholipase C and A2 (244).

Perhaps the mechanism by which peripherally circulating AngII signals to the brain involves prostaglandin production by the cerebral vasculature. These prostaglandins can then diffuse across the blood-brain-barrier to activate efferent sympathetic pathways. This is in contrast to the classically proposed direct action

of AngII on circumventricular organs outside the blood-brain barrier. However this hypothesis remains to be tested.

10.4. Regional sympathetic activation

Enhanced depressor responses to ganglion blockade and significant elevations in plasma NE and whole body NE spillover provides unequivocal proof of global SNS activation in the model of chronic AngII – salt HTN studied in this thesis. However, the specific pattern of sympathetic response is regionally heterogeneous. Sympathetic activation appears to be predominately directed to the splanchnic circulation. In contrast, skeletal muscle sympathetic activity is relatively unchanged and cardiac and renal sympathetic activity seems to be decreased. This regionalized activation is depicted in **figure 37**.

The splanchnic SNS is the critical neural target in chronic AngII - salt HTN. Celiac ganglionectomy (CGx), to selectively disrupt sympathetic innervation to the splanchnic organs, prevents AngII-salt mediated increases in venous smooth muscle tone (139), significantly attenuates AngII-salt hypertension (141) and essentially abolishes the chronic vasoconstrictor responses to AngII in rats consuming a high salt diet (197). In addition, non-hepatic splanchnic vascular resistance is increased. These findings are most consistent with an increase in splanchnic sympathetic activity, although direct assessment of splanchnic nerve activity is required for definitive proof.

Despite the widely held belief that renal sympathetic efferents are the only ones capable of chronically increasing BP (104), the renal nerves did not contribute to the sustained increase in AP in this model of HTN produced by chronic AngII infusion. In fact, surgical removal of the renal sympathetic nerves actually tended to exacerbate the HTN. This finding would be most consistent with a protective role of the renal nerves to buffer the increase in BP, perhaps through a baroreflex mediated reduction in renal sympathetic nerve activity (RSNA). This proposed reduction in RSNA has subsequently been confirmed by chronic renal nerve recordings in this model of AngII HTN by the Osborn laboratory (unpublished). Renal denervation also removes the sensory afferent nerves. The finding that renal denervation exacerbated chronic AngII HTN is also consistent with a loss of sensory nerve mediated vasodilation.

Measurements of heart rate (HR) provide a useful index of cardiac sympathetic activity (135). HR was significantly decreased over the first several days of AngII infusion before returning to control levels within 4-5 days. This probably reflects an initial baroreflex mediated reduction in cardiac sympathetic activity, in response to the initial increase in BP. Cardiac sympathetic activity then appears to return to normal, likely a result of baroreflex resetting. At no time was HR significantly increased from control levels indicating that cardiac SNS activity is unlikely to have increased. Post-ganglionic cardiac sympathetic efferents originate in the paravertebral stellate ganglion. Surgical removal of this ganglion bilaterally would provide more insight into the role of cardiac sympathetic nerves

in the AngII – salt model. This technique has recently been described by the Osborn laboratory.

Measurements of hind-limb NE spillover which predominately reflect sympathetic outflow to skeletal muscle were relatively unchanged by chronic AngII infusion in my studies. This indicates that skeletal muscle is not a major target of AngII induced sympathoactivation in rats.

Esler and colleagues have used regional NE spillover techniques to elucidate the importance of regionalized sympathetic activation in human cardiovascular diseases, including HTN (2, 62, 67, 68, 70). The exact pattern of regional sympathetic activation depends on the population studied. For example, lean essential hypertensives have significant increases in sympathetic activity to the heart, muscle and kidneys, with relatively unchanged levels of splanchnic activity (72). In contrast the aging population has elevated levels of muscle, heart and splanchnic sympathetic activity and unchanged renal activity (72). None-the-less, sympathetic activation patterns are commonly regionally specific in human cardiovascular disease.

What mechanisms underlie regionalized patterns of sympathetic activation? The organotopy hypothesis states that separate groups of barosensitive neurons within the RVLM preferentially control sympathetic outflow to different vascular beds (31, 104, 178, 179). For example one group of neurons predominately

determine sympathetic activity to the kidneys, whereas another group controls the splanchnic circulation (104). The most convincing evidence in support of this hypothesis comes from functional studies in cats where RVLM microstimulation produces activation of different sympathetic nerves, depending on the site of stimulation (31, 104, 178, 179). However, most anatomical attempts to identify discrete populations of neurons that control specific sympathetic responses have been inconclusive (129, 137, 247, 249). Elegant recent studies conducted by Sved and colleagues, using retrograde labeling by pseudorabies virus to track the central origin of sympathetic nerves, documented neurons that selectively innervate one peripheral target (32, 33). Although some of these neurons selectively innervated, for example, the spleen, they were mixed amongst other neurons that selectively innervated, for example, the kidney. This may explain why other anatomical studies have not conclusively documented these populations of cells.

A strict organotopic arrangement of RVLM barosensitive neurons provides a plausible explanation for the ability of AngII to selectively increase splanchnic SNS activity in my studies. It is possible that AngII infusion results in selective activation of osmotically sensitized neurons that principally innervate the splanchnic bed. This would result in an increase in BP and a baroreflex mediated reduction in SNS activity to other beds, including the kidney. What is the physiological rationale for a pathway of selective splanchnic SNS activation by the combination of AngII and salt? It could be speculated that it provides a

beneficial mechanism to compensate hemodynamically in pathological conditions accompanied by increases in plasma AngII and osmolality, such as blood loss and dehydration. Selective sympathetic activation to the high-capacitance splanchnic bed can rapidly mobilize venous stores and restore central blood volume, without the detrimental effects on regional blood flow that would accompany systemic sympathetic mediated vasoconstriction. This assertion is not without basis, as it has been shown that hyperosmolality and AngII interact centrally during controlled and progressive blood loss in sheep to compensate for reduced intravascular volume by improved preservation of cardiac output and a lower total systemic vascular resistance (223). The documented preservation in cardiac output is most likely due to sympathetically mediated active-capacitance responses in splanchnic veins and venules, to translocate venous stores. However, splanchnic SNS activity was not assessed in this study.

HTN that develops in response to AngII infusion in rats fed a high salt may therefore be the result of engaging an evolutionarily conserved neural pathway whose main function is to protect against dehydration and hypovolemia.

10.5. The role of salt in sympathetic responses to AngII

Central to the hypothesis tested in these studies is that the magnitude of SNS activation by AngII infusion would be significantly enhanced by a high-salt diet. However, it was somewhat surprising to discover that SNS activation by AngII was entirely dependent on the presence of a high salt diet. That is, infusion of

AngII alone, in animals fed a normal salt diet, did not produce measurable increases in sympathetic activity, as assessed by depressor responses to ganglion blockade, measurements of plasma NE and NE spillover and regional denervation techniques. The purpose of this thesis was to understand the hemodynamic mechanisms by which SNS activation can chronically increase BP. Salt was exploited as a tool to provide a model in which to do this. The goal of these studies was not to identify the mechanism by which salt potentiates the SNS activating actions of AngII. However, the critical role of salt in the sympathetic responses to AngII is worthy of comment.

Although abundant evidence supports SNS activation as a possible cause of salt-sensitive HTN, the exact mechanism by which salt is acting is unknown. It has been proposed that a high salt diet modestly increases plasma NaCl concentrations, which activates brain osmoreceptors to ultimately increase SNS sensitivity (18). There has been little definitive evidence in support of this hypothesis until recently when Stocker and coworkers convincingly demonstrated that elevated dietary salt enhances the excitability of sympathetic networks emanating from the RVLM (1). The background level of SNS tone is largely determined by the level of activity of neurons within the RVLM (104). Microinjections of L-glutamate into the RVLM were shown to produce significantly greater increases in BP and directly recorded splanchnic and renal sympathetic activity in rats consuming a high salt diet compared to those on standard rat chow (1). It is interesting to note that this enhanced sympathetic sensitivity was

noted after 14 and 21 days of salt intake, but not after 1 or 7 days of salt consumption. In addition, increased SNS sensitivity persisted for several days after the cessation of the salt challenge indicating that these sympathetic responses are likely dependent on a slowly developing and reversible form of neural plasticity (1). Electrolytic lesions of the organum vasculosum of the laminae terminalis (OVLT) attenuate sympathetic responses to central hyperosmolality, likely implicating the OVLT as the site of detection of the osmotic stimulus (238). The exact molecular and cellular mechanisms of the effect of salt on sympathetic activity are yet to be resolved.

Chronic consumption of a high salt diet also significantly enhances RVLM responsiveness to inhibitory GABAergic input (1). This may explain why salt alone does not affect BP or SNS activity in normotensive rats, but only under conditions of enhanced excitatory input. AngII appears to generate an important source of excitatory input to the RVLM under hypertensive conditions (125, 184). Therefore, in my studies chronic consumption of a high salt diet is likely acting to sensitize critical sympathetic brain centers to the excitatory input provided by AngII infusion. In the absence of a high salt diet these brain centers are not sensitized and the excitatory stimulation provided by AngII infusion is not sufficient to activate sympathetic efferent pathways.

10.6. Relevance to Guyton's model of the human circulation

Guyton's well publicized model of the circulation is based on the hypothesis that the long-term level of BP is determined by the renal regulation of total body blood volume in order to generate a pressure that is sufficient to maintain sodium balance (106, 107). Guyton's theory predicts that HTN is the result of a relative decrease in renal sodium and water excretion, which causes volume expansion, tissue over-perfusion and a whole body autoregulatory vasoconstrictor response (105). Similar to hypertensive humans and most experimental models of HTN, blood volume was shown to be unchanged at all time points in my studies of chronic AngII HTN in the rat. Techniques to estimate blood volume are often criticized as not sensitive enough to detect the small changes in volume required to chronically increase BP. However, this is difficult to reconcile with early studies such as that by Frye and Braunwald in 1960 where administration of 1.5 liters of blood to man had no effect on BP or CO (89). These studies highlight the huge capacity of the venous system to tolerate substantial changes in blood volume without a change in arterial pressure; and as noted by Luetscher and colleagues "cast doubt on the theory that volume expansion raises blood pressure by a sequence involving increased cardiac output, overperfusion of tissues, and systemic autoregulation" (166).

In contrast to this major disparity with Guyton's predictions, findings of my thesis work are remarkably consistent with a model of the human circulation proposed by Luetscher and colleagues in 1973 (166) in response to Guyton's pioneering efforts at computer simulation (107). In retrospect, it is rather unfortunate that

Luetscher's model, which emphasizes the importance of BP regulation by the autonomic nervous system and the renin-angiotensin system, was not received as warmly as that proposed by Guyton. Luetscher and co-workers postulated that the autonomic nervous system ultimately controls central blood volume and "so long as the autonomic nervous system is intact, the peripheral capacitance vessels can yield or accept substantial volumes of blood without significant effects on cardiac output or arterial pressure" (166). In contrast to Guyton, in Luetscher's model of AngII dependent HTN elevations in blood volume are not assumed, and CO is supported by enhanced venous return as a result of constricted peripheral capacitance vessels (166).

The accord between Leuetscher's predictions and my results are uncanny. In complete agreement with his proposed model, my thesis studies suggest that sympathetically mediated venoconstriction increases central blood volume and is the predominant mechanism increasing BP in chronic AngII-salt HTN.

The volume related mechanisms of HTN proposed by Guyton cannot be entirely ruled out based on my work. Firstly, the techniques available for the measurement of blood volume are not capable of detecting subtle changes. It has been determined that volume estimates made by the Evans blue dilution method are within 10% of the actual volume. It is possible that small increases in blood volume are occurring in response to chronic infusion of AngII but are not detectable with the techniques I employed. A modest expansion of blood volume

is likely to be of considerable hemodynamic consequence in an experimental model of HTN associated with a reduction in vascular capacitance. Most likely, sympathetically mediated venoconstriction is working together with a reduction in renal sodium and water excretion to cause chronic AngII-salt HTN in rats. Secondly, only one model of HTN was studied in my thesis and it is almost certain that different mechanisms operate to chronically increase BP in other models of experimental HTN. Volume expansion may be of particular importance under other conditions.

10.7. Overall significance, perspectives and therapeutic implications

My thesis work has documented a novel role for the sympathetic control of the high-capacitance splanchnic circulation in mediating long-term changes in BP. This significantly improves our current understanding of sympathetic mechanisms leading to HTN.

Compelling evidence has been provided that SNS activation is a mechanism responsible for salt-sensitive HTN. Therefore, in general, sympathoinhibition may be applied as a successful therapeutic strategy to lower AP in salt-sensitive human HTN. More specifically the splanchnic SNS represents an attractive peripheral site which could be targeted by novel sympatholytic drugs in an attempt to avoid undesirable side-effects that often accompany centrally acting pharmacologic agents. These studies have also established cyclooxygenase derived inflammatory products as critical signals in activating the SNS in salt-

sensitive HTN. Therefore, identifying the specific vasoactive prostanoid which possess sympathoactivating properties provides an additional area of therapeutic promise. Finally, I have presented evidence that AngII exacerbates stress responses. This provides conceptual support to the recently proposed notion that pharmacological antagonism of the renin-angiotensin system may be a useful in the treatment of stress-related disorders (224).

Very recently it has been advocated by Biaggioni that review of experimental and human studies indicate that the SNS should be considered as a target in the treatment of obesity-associated HTN (13). It is my hope that the work presented in this thesis contribute to a similar platform of evidence to provide incentive to develop novel sympatholytic drugs for the treatment of salt-sensitive HTN.

10.8. Future direction

The most pressing question remaining from this work is; can direct measures of splanchnic sympathetic activity definitively confirm increased regionally selective splanchnic sympathoactivation in response to chronic AngII infusion in rats fed a high salt diet. Application of the complementary techniques of microneurography and regional NE spillover, while technically challenging, provide the only way to address this issue. Central to the conclusions of this work is that sympathetically mediated venoconstriction results in an increase in central blood volume. Optimizing measurements of regional body fluid distribution using bioimpedance methods will allow this hypothesis to be more thoroughly tested.

In relation to the therapeutic implications discussed earlier, a troubling caveat is that many interventions that prevent the development of HTN are not equally effective at reducing BP, once the HTN has been established. In this regard it is critical to determine if selective splanchnic denervation and cyclooxygenase inhibition are efficacious in reducing BP in established AngII-salt HTN. Finally, dissecting out the prostanoid responsible for AngII mediated SNS activation, and its primary tissue targets, provides a promising area of research to explore.

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